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Minireview

MINIREVIEW

Bordetella filamentous hemagglutinin and fimbriae: critical adhesins with unrealized vaccine potential

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One sentence summary: Bordetella fimbriae and filamentous hemagglutinin are critical adhesins and are potential protective antigens for next-generation vaccines.

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ABSTRACT

Pertussis, or whooping cough, is a highly contagious respiratory disease that is caused by the Gram-negative bacterium *Bordetella pertussis,* which is transmitted exclusively from human to human. While vaccination against *B. pertussis* has been successful, replacement of the whole cell vaccine with an acellular component vaccine has correlated with reemergence of the disease, especially in adolescents and infants. Based on their presumed importance in mediating adherence to host tissues, filamentous hemagglutinin (FHA) and fimbria (FIM) were selected as components of most acellular pertussis vaccines. In this review, we describe the biogenesis of FHA and FIM, recent data that show that these factors do, in fact, play critical roles in adherence to respiratory epithelium, and evidence that they also contribute to persistence in the lower respiratory tract by modulating the host immune response. We also discuss shortcomings of whole cell and acellular pertussis vaccines and the possibility that FHA and FIM could serve as effective protective antigens in next-generation vaccines.

Keywords: *Bordetella pertussis*; filamentous hemagglutinin; fimbriae; immunity

INTRODUCTION

Pertussis (aka whooping cough) is caused by the fastidious Gram-negative bacterium *Bordetella pertussis* (Melvin *et al.* 2014). This highly contagious respiratory disease is most severe in infants and young children. Pertussis was widespread until the late 1940s, with annual case rates as high as 270 000 in the United States and fatality rates at about 10% (Clark 2014). Whole cell vaccines (wP), composed of killed *B. pertussis* organisms and introduced in the late 1940s, were hugely successful and morbidity and mortality rates plummeted throughout the 1950s and 1960s in areas with high vaccine coverage (Clark 2014). Adverse publicity in the 1970s, however, led to decreased compliance and prompted the development of acellular component vaccines (aP) that are composed of one to five purified pertussis proteins [chemically inactivated pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (PRN), and/or fimbriae of serotype 2 or 3 (FIM2, FIM3)]. aP vaccines were introduced in the 1990s and are the only pertussis vaccines currently licensed in the USA and most other developed countries. Coincident with the transition to the aP vaccines, case rates and regional pertussis epidemics have increased steadily, with pertussis disease incidence in 2012 reaching a level last seen in the 1950s (Clark 2014). Recent disease rates have increased most dramatically in adolescents and epidemiological studies indicate that immunity from aP vaccines is not as durable as initially thought (Klein *et al.* 2012; Misegades *et al.* 2012; Witt, Katz and Witt 2012; Tartof *et al.* 2013; Koepke *et al.* 2014; McGirr and Fisman 2015). Consequently, there

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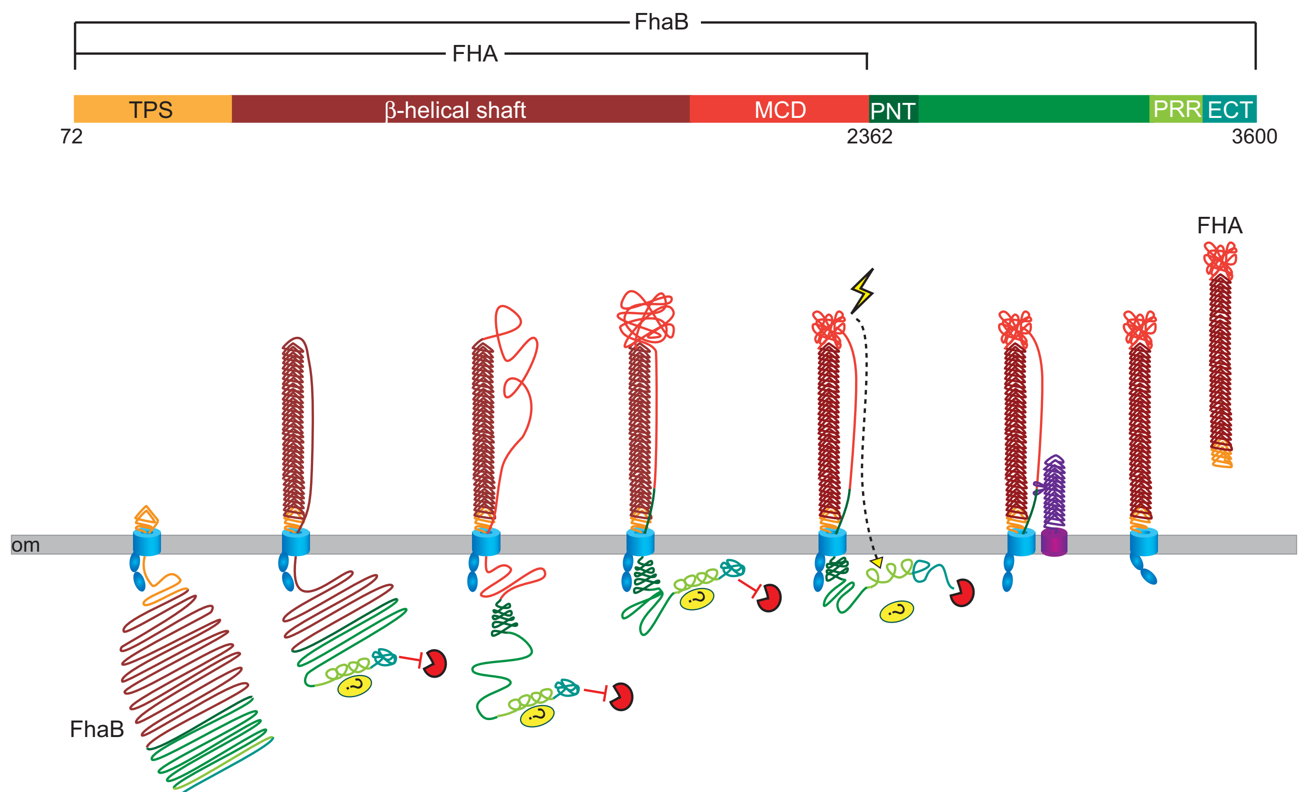

Figure 1. FHA biogenesis. FhaB is an ~370 kDa polypeptide. After removal of the 71 aa signal sequence, the TPS domain of FhaB (gold) interacts with the POTRA domains of FhaC (blue), initiating translocation across the outer membrane (om). The N-terminal ~2500 aa of FhaB fold into a β-helix on the cell surface. The C-terminal proline-rich region (PRR) may interact with a periplasmic protein or protein domain, and the extreme C-terminal domain (ECT) inhibits an unknown protease. The N-terminus of the prodomain (PNT, dark green) prevents translocation of the prodomain across the om. In response to an unknown signal, the ECT releases inhibition of the unknown protease and the prodomain is degraded. FhaB is also processed by the serine protease SphB1 (purple) to form 'mature' ~240 kDa FHA. FHA is released from the cell surface by an unknown mechanism.

is renewed interest in understanding molecular mechanisms of pertussis pathogenesis and the immune response that is generated upon natural infection with the goal of developing a new generation of safe yet effective pertussis vaccines.

Two *B. pertussis* surface structures that have featured prominently in the development of pertussis vaccines are FHA and FIM. Both were identified initially as hemagglutinins or agglutinogens responsible for determining the serotype of *B. pertussis* strains. Agglutinogens 2 and 3 were subsequently determined to be pili or fimbriae (FIM2 and FIM3) and FHA was determined to be a large non-fimbrial filamentous hemagglutinating molecule (Ashworth, Irons and Dowsett 2006). Despite little supportive data at the time, both FHA and FIM were presumed to mediate adherence of *B. pertussis* to host tissues and therefore there was an effort to ensure that wP vaccines were made from *B. pertussis* strains producing both FIM2 and FIM3 (as well as FHA) and that aP vaccines included these antigens. In this review, we discuss current knowledge of the function of FHA and FIM in the ability of *B. pertussis* to establish respiratory infection and to influence the development of immunity and considerations for the development of new pertussis vaccines. Although most studies have been conducted using *B. pertussis* or proteins derived from *B. pertussis*, the human-restricted host range of this organism has hampered studies aimed at determining the roles of specific virulence factors in disease pathogenesis and the induction of immunity. Several groups have therefore included *B. bronchiseptica* in their analyses because this extremely closely related organism naturally infects nearly all mammals and therefore pathogenesis can be investigated in the context of a natural bacteria–host interaction. Our review will therefore also summarize data obtained from studies with *B. bronchiseptica*.

FHA AND FIM BIOGENESIS

Electron microscopy and structural analyses indicate that FHA, which is both cell associated and released into culture supernatants, is an ~4 × 40 nm β-helix with a globular domain at one end and a molecular mass of ~240 kDa (Makhov *et al.* 1994; Kajava *et al.* 2001). FHA is exported to the cell surface by the two-partner secretion (TPS) pathway. TPS is a broadly used mechanism by which Gram-negative bacteria export large, β-helical, exoproteins to the cell surface using outer membrane, channel-forming, β-barrel proteins and FHA and its cognate outer membrane transporter protein, FhaC, are its prototypical members (Mazar and Cotter 2007; Jacob-Dubuisson *et al.* 2013). FHA is first synthesized as an ~375 kDa preproprotein called FhaB (Fig. 1). It is translocated across the cytoplasmic membrane by the general Sec system via a signal sequence that contains an extended signal peptide region (ESPR) at its N-terminus (Chevalier *et al.* 2004). ESPRs are present on many TPS exoproteins and autotransporter proteins and although they appear to be important for controlled secretion of these large proteins, their exact function is unclear (Desvaux *et al.* 2006, 2007). After removal of the ESPR-containing signal sequence, the N-terminal ~250 aa 'TPS domain' of FhaB initiates export across the outer membrane by interacting with the periplasmically located polypeptide transport

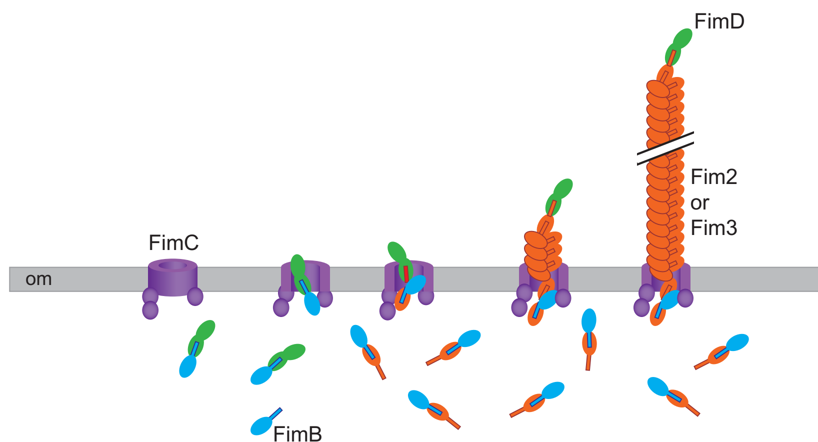

Figure 2. FIM biogenesis. The FimC protein is predicted to be an integral outer membrane (om) β-barrel usher protein. FimB is predicted to function as a chaperone that binds FimD, Fim2 and Fim3 proteins, preventing their degradation in the periplasm and directing them to FimC for translocation across the om. Within FimC, the FimB chaperone transfers the fimbrial subunit to the next subunit in the channel by donor strand exchange, resulting in elongation of the FIM structure.

associated (POTRA) domains of FhaC (Hodak *et al.* 2006; Delattre *et al.* 2011; Baud *et al.* 2014). Export of FhaB proceeds in an N- to C-terminal direction, with the N-terminus remaining associated with FhaC such that a hairpin structure forms and elongates on the cell surface (Mazar and Cotter 2006). All TPS exoproteins are predicted to contain large β-helical regions, especially near their N-termini, and it has been postulated that folding of the stable β-helix facilitates export by contributing to a Brownian ratchet mechanism (Jacob-Dubuisson *et al.* 2013). Once approximately 2500 aa of FhaB have been exported to the surface, a region called the prodomain N-terminus (PNT) prevents further translocation and hence the C-terminal ~1200 aa of FhaB (called the prodomain) remains in the periplasm (Noël *et al.* 2012). Specific subdomains within the prodomain are important for preventing degradation by an unknown protease, influencing the folding of the C-terminal domain of mature FHA (called the MCD) on the cell surface, and contributing somehow to resistance to phagocytic cell clearance (Noël *et al.* 2012; Melvin *et al.* 2015) (discussed below). Degradation of the prodomain in the periplasm and cleavage N-terminal to the prodomain by the surface-localized autotransporter SphB1 results in the formation of the mature ~240 kDa FHA molecule that is oriented with its C-terminus (the MCD) located distally from the cell surface (Coutte *et al.* 2001; Mazar and Cotter 2006). A substantial amount of FHA is ultimately released from the cell. Neither the mechanism of FHA release nor whether FHA release is important for infection is known.

Bordetella FIM are members of the type 1 pili family. These long, thin, hair-like structures are composed of thousands of pilin subunits. The structures are ~7.5 nm in diameter and can be several hundred nanometers in length (Heck, Trus and Steven 1996). Type I pili are exported by the chaperone–usher mechanism, which has been extensively studied in uropathogenic *Escherichia coli* (UPEC) (see Busch, Phan and Waksman 2015 for a review). Based on aa sequence similarity, the *Bordetella* FimB, FimC and FimD proteins are hypothesized to function as the periplasmic chaperone, the outer membrane usher and the tip adhesin, respectively (Willems, van der Heide and Mooi 1992). The major pilins in *Bordetella* are Fim2 and Fim3. Other major pilins (FimX, FimN and FimA) are also encoded by most strains, but their production has not been detected. According to the current model based on UPEC pili (Busch, Phan and Waksman 2015), the pilin, chaperone and usher proteins are translocated across the cytoplasmic membrane by the Sec system. The pilin subunits bind to the chaperone, FimB in *Bordetella*, by a mechanism called donor strand complementation in which an N-terminal extension (Nte) region of the pilin fits within a groove in the chaperone to complete an immunoglobulin-like domain. The chaperone delivers the pilin subunits to the usher, where donor strand exchange (transfer of the Nte of the pilin from the chaperone to another pilin protein) and translocation of a growing chain of pilin subunits across the outer membrane occur (Fig. 2). The helical nature of the pilus rod is thought to contribute to the ability of the pili to withstand shear forces while maintaining adhesion because although it is rigid, it can uncoil under stress, acting like a spring or shock absorber (Thanassi, Bliska and Christie 2012). This feature would likely be important during adherence of *Bordetella* to beating cilia in the trachea and bronchi.

Genes encoding the exoproteins and outer membrane transporters of most TPS systems are adjacent and cotranscribed (Jacob-Dubuisson *et al.* 2013). Similarly, genes required for type 1 pili biogenesis are typically clustered and coregulated (Busch, Phan and Waksman 2015). In *Bordetella*, however, the *fimA*, *fimB*, *fimC* and *fimD* genes are located between *fhaB* and *fhaC* (Fig. 3). Moreover, *fimD* and *fhaC* overlap such that they are both transcriptionally and translationally coupled. A single promoter 5′ to the *fimB* gene appears to be responsible for transcription of the *fimBCDfhaC* operon (Willems, van der Heide and Mooi 1992). While the *fimA* gene is intact in *B. bronchiseptica* (and *B. parapertussis*) strains, it is missing its 5′ end and promoter region in *B. pertussis* strains (Fig. 3 and Boschwitz *et al.* 1997). Although *fimA* is predicted to encode a major pilin subunit, FimA-containing FIM have not been described. All *B. pertussis* and *B. bronchiseptica* strains that have been characterized produce FIM composed of Fim2, Fim3 or both, the structural genes for which are located elsewhere on the chromosome. The *fhaB-fimA-fimBCDfhaC* gene cluster is adjacent to and divergent from the *bvgAS* operon, which encodes the BvgAS two-component regulatory system that activates transcription of all known protein virulence factor-encoding genes (Cotter and DiRita 2000; Cummings *et al.* 2006). When the BvgAS system is active (such as when the bacteria are within the mammalian respiratory tract; Cotter and Miller 1994; Merkel *et al.* 1998), BvgA is phosphorylated and activates transcription of the *fhaB* and *fimB* promoters, as well as the *bvgAS* P1 promoter, by binding high-affinity binding sites (Boucher *et al.* 1997, 2003; Cotter and Jones 2003).

Figure 3. The *bvgAS-fhaB-fimA-fimBCDfhaC* gene cluster. The arrangement of the *bvgAS-fhaB-fimA-fimBCDfhaC* genes on the chromosome is shown. The pointed end of the box for each gene represents the 3′ end. Promoters are shown as arrows.

Transcription of the *fim2* and *fim3* genes is also activated by BvgAS (Chen *et al.* 2010). Hence, FHA and FIM are amongst the first virulence factors produced when the bacteria move from Bvg$^-$ phase conditions (*ex vivo*) to Bvg$^+$ phase conditions (*in vivo*). The fact that the *bvgAS, fhaB* and *fimBCDfhaC* genes are clustered and coregulated suggests that they play similarly important and perhaps codependent or coordinated roles during infection.

FHA AND FIM FUNCTION

The locus responsible for FHA production was first identified in 1983 as the site of a Tn5 transposon in a *B. pertussis* mutant that was unable to agglutinate sheep erythrocytes (Weiss *et al.* 1983). Comparison of this strain, or strains containing deletion mutations in *fhaB,* with wild-type *B. pertussis* for their ability to adhere to ciliated epithelium (Relman *et al.* 1989; Prasad *et al.* 1993), non-ciliated epithelium (van den Berg *et al.* 1999), Chinese hamster ovary cells (Relman *et al.* 1989; Aricò *et al.* 1993), monocytes (Relman *et al.* 1989; Prasad *et al.* 1993; Ishibashi, Claus and Relman 1994) and neutrophils (Mobberley-Schuman and Weiss 2005) suggested that FHA plays an important role in mediating adherence of the bacteria to host cells. Similarly, studies with strains containing either a large deletion mutation in *fhaB* or in which the *fhaB* and *fhaC* genes were expressed in a Δ*bvgS* mutant indicated that FHA is both necessary and sufficient for *B. bronchiseptica* to adhere to a variety of cells lines *in vitro* (Cotter *et al.* 1998; Mattoo, Miller and Cotter 2000). Although early preparations of anti-FHA antibodies often contained antibodies against other *Bordetella* factors, blocking studies also implicated FHA as an important adhesin *in vitro* (Sato *et al.* 1981; Redhead 1985; Prasad *et al.* 1993; van den Berg *et al.* 1999; Julio *et al.* 2009).

Analyses aimed at identifying domains within FHA that mediate interactions with host cells initially implicated a region near the N-terminus of FHA (named the heparin-binding domain, HBD) in binding sulfated carbohydrates such as heparin and haparan sulfate and in agglutinating sheep erythrocytes. This region contains consensus heparin-binding sites of fibronectin (Cardin *et al.* 1991; Busby *et al.* 1995) and FHA purified from culture supernatants was shown to interact with heparin-sepharose (Menozzi *et al.* 1994). Moreover, incubation with heparin decreased *B. pertussis* adherence to HeLa cells (Menozzi *et al.* 1994). However, the current model for FHA secretion places the HBD near the bacterial surface where it would likely be inaccessible to host cell molecules (Mazar and Cotter 2006; Noël *et al.* 2012), casting doubt about whether these interactions occur *in vivo*. Residues 1141–1279 were named the carbohydrate recognition domain because a monoclonal antibody that recognized an epitope in this region blocked the binding of FHA to glycolipids and reduced binding of *B. pertussis* to rabbit ciliated epithelial cells, which could also be blocked by glycolipids (Tuomanen *et al.* 1988; Prasad *et al.* 1993). A polyclonal antibody raised against a polypeptide containing this region did not, however, block binding of *B. pertussis* or *B. bronchiseptica* to either rat lung epithelial (L2) cells or J774A.1 macrophage-like cells (Julio *et al.* 2009). FHA also contains an RGD triplet centered at aa 1098 (numbering based on FHA from *B. pertussis*) which was implicated in adherence to monocytes (Relman *et al.* 1990; Aricò *et al.* 1993; Ishibashi, Claus and Relman 1994) by interacting with leukocyte response integrin-integrin associated protein (LRI-IAP) complexes and inducing surface presentation of CR3 (aka CD11b/CD18) (Ishibashi, Claus and Relman 1994). However, a *B. bronchiseptica* strain producing FHA with RAE in place of RGD was indistinguishable from wild-type bacteria in its ability to establish persistent infection in rats and mice (Julio *et al.* 2009). Focus on the N-terminal regions of FHA was due in part to the assumption that FHA was oriented with its N-terminus extended distally from the cell surface. Secretion and topology studies in 2006 (Mazar and Cotter 2006), however, showed that the N-terminus remains anchored at the cell surface and the C-terminus of mature FHA (the MCD) is located distally, placing it in an optimal location to interact with host cells. Antibody blocking studies and mutational analyses demonstrated the importance of the MCD in binding to epithelial cells and macrophages *in vitro* and for FHA function *in vivo* (Julio *et al.* 2009), and therefore it seems likely that FHA mediates interactions with host cells during infection primarily via the MCD, although roles for other regions of FHA have not been definitively ruled out. Identifying the molecule(s) with which FHA interacts *in vivo* remains an important and elusive goal.

Although fimbriae have been shown to function as important adhesins for many bacterial pathogens, evidence for FIM mediated-adherence in *Bordetella* has been lacking. Several studies investigating the role of FIM in *B. pertussis* were conducted in the 1990s (Hazenbos *et al.* 1994, 1995; Geuijen *et al.* 1997), but all of the strains used in these experiments were also defective for FHA production and hence conclusions about the role of FIM could not be drawn. Rodriguez *et al.* (2006) reported that anti-FIM antibodies could block attachment of *B. pertussis* to epithelial cells, but FIM-deficient bacteria were not included in the analysis as a control. A *B. bronchiseptica* strain lacking FIM due to an in-frame deletion mutation of the *fimBCD* genes but unaltered for FHA production was indistinguishable from wild-type bacteria in its ability to adhere to a broad range of cell lines *in vitro*, providing evidence against a role for FIM in adherence (Mattoo, Miller and Cotter 2000). However, this strain was defective in adherence to ciliated rabbit tracheal explants, suggesting that FIM may mediate adherence specifically to cilia (Edwards, Groathouse and Boitano 2005). Given the lack of evidence for FIM-mediated adherence *in vitro*, it is not surprising that a host cell receptor has not been identified. While studies using purified fimbrial subunits suggested that FIM may interact with sulfated sugars (Geuijen *et al.* 1998), confirmatory experiments with intact fimbriae have not been reported.

To avoid caveats associated with working with cultured cell lines *in vitro* and to investigate the role of FIM and FHA in adherence to respiratory epithelium in a biologically relevant context, we recently developed an *in vivo* adherence assay using *B. bronchiseptica* and mice (Scheller *et al.* 2015). We inoculated mice with 50 μl of PBS containing 7.5×10^4 cfu of wild-type or mutant bacteria, performed bronchoalveolar lavage (BAL) 30 minutes later,

and determined the number of CFU recovered in the BAL fluid and retained in the lungs post-BAL. These experiments revealed equally critical roles for FHA and FIM in adherence to tissue within the lower respiratory tract; neither factor could mediate adherence without the other as the Δ*fhaB* and Δ*fimBCD* mutants were as defective as each other, as a Δ*fhaB*Δ*fimBCD* double mutant, and as a Δ*bvgS* mutant in this assay. Determining where wild-type, Δ*fhaB* and Δ*fimBCD,* mutants localized within the lungs when not removed by BAL provided additional insight into FHA and FIM function. FHA-deficient bacteria, like wild-type bacteria, localized to major airways (bronchi and bronchioles), while FIM-deficient bacteria localized predominantly to alveoli. These data suggest that FIM mediates adherence specifically to ciliated epithelium (without FIM, the inoculated bacteria bypass the bronchi and bronchioles and reach the alveoli), but that this adherence alone is not sufficient to resist removal by BAL. Similarly, FHA-mediated adherence in the alveoli either does not occur or is insufficiently strong to resist BAL. Hence, we propose a model in which FIM adhere specifically, but with low or moderate affinity, to cilia, bringing the bacteria close enough for FHA to bind to these cells with high affinity that is sufficient for the bacteria to resist BAL in our experiments and mucociliary clearance during infection.

Roles for FIM and FHA in adherence to ciliated epithelium in the lower respiratory tract are consistent with the data obtained from inoculating rats and pigs intranasally with a small volume of PBS containing a relatively small number of bacteria. In these natural-host colonization models, FHA-deficient and FIM-deficient *B. bronchiseptica* are recovered only from the nasal cavity (the initial site of inoculation), while wild-type bacteria colonize the nasal cavity initially, then the trachea within about 5 days and they persist in both the nasal cavity and the lower respiratory tract (predominantly the trachea) indefinitely (Cotter *et al.* 1998; Mattoo, Miller and Cotter 2000; Nicholson, Brockmeier and Loving 2009). Experiments with high-dose, large-volume-inoculation mouse models suggest that FIM and FHA perform functions beyond simply mediating adherence to host cells. Both Δ*fhaB* and Δ*fimBCD* mutants induce a more robust inflammatory response, characterized by high levels of the proinflammatory cytokine interleukin (IL)-1β, and the neutrophil and macrophage chemokines KC and MCP-1, respectively, in the lungs at 1–3 days post-inoculation compared with wild-type bacteria (Henderson *et al.* 2012; Scheller *et al.* 2015). Examination of hematoxylin and eosin-stained lung sections reveal correspondingly increased inflammatory cell infiltrate in the lungs of mice inoculated with the mutants compared with those from mice inoculated with wild-type bacteria. In addition, the Δ*fhaB* and Δ*fimBCD* mutants are cleared from the lungs within 2 weeks post-inoculation while wild-type bacteria typically persist for a month or more in this model. These data suggest that FIM and FHA are required for wild-type *B. bronchiseptica* to suppress the initial inflammatory response to infection and that doing so facilitates their persistence. Coinoculation experiments suggest that in addition to influencing the intensity of the inflammatory response that develops, FHA and FIM also contribute to the ability of *B. bronchiseptica* to defend itself from clearance by the elicited innate immune response (Inatsuka, Julio and Cotter 2005; Henderson *et al.* 2012; Scheller *et al.* 2015). When wild-type and Δ*fhaB* bacteria were coinoculated into mice, the level of proinflammatory cytokines and chemokines present in the lungs 1–3 days post-inoculation was similar to that of mice inoculated with only wild-type bacteria, suggesting that the wild-type bacteria suppressed the overall inflammatory response. However, although the Δ*fhaB* mutant persisted longer in the coinoculation experiment than when inoculated alone, it was still cleared faster than the coinoculated wild-type bacteria, suggesting that it is defective at defending itself against clearance by the recruited phagocytic cells or other components of the early immune response. Coinoculation of wild-type and FIM-deficient bacteria also resulted in proinflammatory cytokine and chemokine levels similar to those induced by wild-type bacteria, but the persistence defect of the Δ*fimBCD* strain was as severe as when inoculated alone. Considering the results of the bacterial localization studies, these data suggest that complementation by wild-type bacteria requires close proximity; wild-type bacteria could 'rescue' the ΔFHA mutant because they also localize to major airways while wild-type bacteria were unable to rescue ΔFIM mutants because the ΔFIM mutants localize to alveoli.

Most recently, we found evidence that it is full-length FhaB, rather than mature ~240 kDa FHA, that is important for mediating resistance to clearance by the elicited inflammatory response (Melvin *et al.* 2015). We constructed and characterized *B. bronchiseptica* strains that produce FhaB proteins lacking either the PRR, the ECT or both of these domains that are located at the end of the full-length FhaB protein (Fig. 1). The ΔPRR strain was indistinguishable from wild-type bacteria in its ability to produce full-length FhaB (aside from the FhaB protein missing its PRR domain in the mutant), to process FhaB to FHA and to release FHA into culture supernatants *in vitro*. The ΔPRR strain was also indistinguishable from wild-type bacteria in its ability to mediate adherence to cell lines *in vitro* and to respiratory epithelium *in vivo* and it suppressed the initial inflammatory response to infection similarly to wild-type bacteria. The ΔPRR mutant was defective, however, in its ability to persist in the lower respiratory tract; it was cleared from the lungs of mice as quickly as the Δ*fhaB* strain. Because this mutant produces mature FHA that is indistinguishable from that produced by wild-type bacteria in our assays, and because the FhaB prodomain is degraded rapidly and does not exist as a stand-alone polypeptide, these results implicate a role for full-length FhaB in persistence, specifically in resistance to clearance by the early immune response. We hypothesize that the PRR domain is involved in delivering a toxin (perhaps adenylate cyclase toxin) to phagocytic inflammatory cells in response to binding that is mediated by the MCD region of full-length FhaB.

Overall, data from comparing wild-type and mutant *B. bronchiseptica* strains in natural-host animal models support a model in which wild-type bacteria use FIM for initial adherence to ciliated epithelial cells in the lower respiratory tract and then FHA for tight adherence to these cells. Colonization of the respiratory epithelium induces inflammation, likely due to recognition of LPS and other MAMPs on, or released by, the bacteria by TLRs on host cells, resulting in the production of proinflammatory cytokines and chemokines. Neutrophils, macrophages, dendritic cells and lymphocytes are then recruited to the site of infection and as these cells attempt to clear the bacteria, they may also release cytokines and chemokines, amplifying the inflammatory response. Wild-type *B. bronchiseptica* (and likely also *B. pertussis*) suppress the inflammatory response in a manner dependent on FIM and FHA, but whether they do so through interactions with epithelial cells, inflammatory cells or both and the mechanisms involved are unknown. Wild-type bacteria, in a manner dependent on the PRR of FhaB, also appear to be capable, to some extent, of resisting clearance by recruited phagocytic cells, which contributes to bacterial persistence in the lower respiratory tract (Melvin *et al.* 2015). The bacteria are ultimately cleared, however, in a manner dependent on the adaptive immune response (Harvill *et al.* 1999).

FHA AND FIM: PROTECTIVE ANTIGENS?

Antibodies were initially thought to be of prime importance for protection against pertussis. Consequently, with the rationale that antibodies against adhesins would block colonization, there was an effort to use *B. pertussis* strains producing both FIM2 and FIM3 in the production of whole cell vaccines and when acellular vaccines were developed, FIM and FHA were considered to be important, if not essential, components. However, the aP and wP vaccines induce serum antibody responses to vaccine components that are similar in magnitude to those induced by infection, yet human case control and cohort studies indicate that immunity induced by aP vaccination is not as durable as immunity induced by wP vaccination, which is not as durable as immunity induced by infection with *B. pertussis*, (Plotkin 2013; Edwards and Berbers 2014; Acosta *et al.* 2015). Moreover, serological correlates of protection were never established in clinical vaccine trials. Attention has therefore turned to understanding the role of cell-mediated immunity in controlling pertussis. These studies have shown that immunization with aP vaccines induces primarily a Th2 or mixed Th1/Th2 response, while immunization with wP vaccines and infection with *B. pertussis* induce primarily a Th1 response (Zepp *et al.* 1996; Ausiello *et al.* 1997, 1999; He *et al.* 1998; Ryan *et al.* 1998; Edwards and Berbers 2014). The hypothesis that a Th1, and possibly a Th17, type cell-mediated immune response is required for protection from pertussis is also supported by data from mouse studies (Mills *et al.* 1993; Barbic *et al.* 1997; Leef *et al.* 2000; Ross *et al.* 2013). Because most of the human studies were conducted before the appreciation of Th17 cells, the induction of this T-cell subpopulation in response to wP vaccination or *B. pertussis* infection in humans is unknown. Recent studies, however, attempted but failed to detect *B. pertussis* antigen-specific Th17 cells in children immunized with aP vaccines, supporting the conclusion that aP vaccines induce exclusively Th2 or Th1/Th2 responses (Stenger *et al.* 2010; Schure *et al.* 2012, 2013) and strengthening the hypothesis that cell-mediated immunity, and not antibodies, is essential for protection.

Insight into the specific contributions of types of immune responses induced by vaccination versus infection has come from studies using the recently developed baboon model. This model, using *Papio anubis*, accurately recapitulates many features of human pertussis, including paroxysmal cough, lymphocytosis and aerosol transmission (Warfel, Beren and Merkel 2012a,b). Baboons infected with *B. pertussis* developed strong, long-lasting Th1 and Th17 responses (Warfel *et al.* 2012b; Warfel, Zimmerman and Merkel 2014). After recovery from the initial infection, the baboons were completely protected from both disease (cough and lymphocytosis) and colonization upon challenge with *B. pertussis*. Baboons immunized with wP (in the form of DTwP) also developed both Th1 and Th17 responses, although they were not as strong as those induced in response to infection (Warfel, Zimmerman and Merkel 2014). DTwP-vaccinated baboons were protected from disease, but not from colonization, although they cleared infection in about half the time as naïve animals (~2 weeks versus 1 month). Baboons immunized with DTaP developed a mixed Th1/Th2 response. They were also protected from disease, but not colonization; in fact, they cleared infection as slowly as naïve animals. Most importantly and perhaps revealing with regard to the current reemergence of pertussis, baboons immunized with DTaP and then challenged were capable of transmitting infection to naïve animals. All baboons that were either infected with *B. pertussis* or immunized with wP or aP vaccines generated high titers of serum anti-*Bordetella* antibodies, demonstrating that, consistent with the studies on humans, differences in serum antibody titer do not account for differences in protection. Warfel and colleagues concluded from the baboon (and human) studies that antibodies may mediate protection against disease while Th1 and Th17 type cell-mediated immune responses mediate protection against colonization (Warfel, Zimmerman and Merkel 2014; Warfel and Edwards 2015). While this explanation is plausible, it does not fully account for the fact that baboons that had been infected previously were completely protected from colonization while baboons vaccinated with wP vaccines were not, yet both had generated Th1 and Th17 responses. It is possible that the difference in protection from colonization in convalescent and wP-vaccinated animals stems from differences in the magnitude of the Th1 and Th17 responses induced, or differences in the specific antigens to which these responses were directed. However, another possibility that has not been investigated is that protection from colonization is mediated by mucosal IgA antibodies and that parenteral immunization with either aP or wP vaccines fails to generate a sufficiently strong mucosal antibody response to afford this protection. Our recent data demonstrating the essentiality of FHA and FIM for adherence to respiratory epithelium suggest that mucosal antibodies against either or both of these factors would block adherence and therefore protect against colonization and, consequently, disease. In support of this hypothesis, two studies using an intranasal inoculation protocol have shown that FHA could induce a protective immune response in mice (Alonso *et al.* 2002; Knight *et al.* 2006). We therefore believe that efforts towards developing safe and efficacious pertussis vaccines should include obtaining a complete understanding of the immune responses that are induced by natural infection as well as those induced by wP and aP vaccines for comparison and that these studies should include measurements of mucosal IgA levels. A finding that mucosal antibodies to FHA and /or FIM are sufficient for protection against colonization would have a dramatic impact on vaccine development as effort could be focused on inducing this specific type of response, perhaps even focused on specific domains of FHA and FIM.

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