New Insight into Filamentous Hemagglutinin Secretion Reveals a Role for Full-Length FhaB in *Bordetella* Virulence

Jeffrey A. Melvin,* Erich V. Scheller, Christopher R. Noël,* Peggy A. Cotter

Department of Microbiology and Immunology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

* Present address: Jeffrey A. Melvin, Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA; Christopher R. Noël, MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan, USA.

**ABSTRACT** *Bordetella* filamentous hemagglutinin (FHA), a primary component of acellular pertussis vaccines, contributes to virulence, but how it functions mechanistically is unclear. FHA is first synthesized as an ~370-kDa preproprotein called FhaB. Removal of an N-terminal signal peptide and a large C-terminal prodomain (PD) during secretion results in "mature" ~250-kDa FHA, which has been assumed to be the biologically active form of the protein. Deletion of two C-terminal subdomains of FHA did not affect production of functional FHA, and the mutant strains were indistinguishable from wild-type bacteria for their ability to adhere to the lower respiratory tract and to suppress inflammation in the lungs of mice. However, the mutant strains, which produced altered FhaB molecules, were eliminated from the lower respiratory tract much faster than wild-type *B. bronchiseptica*, suggesting a defect in resistance to early immune-mediated clearance. Our results revealed, unexpectedly, that full-length FhaB plays a critical role in *B. bronchiseptica* persistence in the lower respiratory tract.

**IMPORTANCE** The *Bordetella* filamentous hemagglutinin (FHA) is a primary component of the acellular pertussis vaccine and an important virulence factor. FHA is initially produced as a large protein that is processed during secretion to the bacterial surface. As with most processed proteins, the mature form of FHA has been assumed to be the functional form of the protein. However, our results indicate that the full-length form plays an essential role in virulence in *vivo*. Furthermore, we have found that FHA contains intramolecular regulators of processing and that this control of processing is integral to its virulence activities. This report highlights the advantage of studying protein maturation and function simultaneously, as a role for the full-length form of FHA was evident only from *in vivo* infection studies and not from *in vitro* studies on the production or maturation of FHA or even from *in vitro* virulence-associated activity assays.

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 currently reemerging 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: 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 (8–11). 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 infection and persistence in the lower respiratory tracts of mice, rats, and pigs by *Bordetella bronchiseptica* (2, 6, 12–15), 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

---

**Citation** Melvin JA, Scheller EV, Noël CR, Cotter PA. 2015. New insight into filamentous hemagglutinin secretion reveals a role for full-length FhaB in *Bordetella* virulence. mBio 6(4):e01189-15. doi:10.1128/mBio.01189-15.
B. pertussis and B. bronchiseptica in animal models of disease (13, 16, 17). For these reasons, we use B. bronchiseptica infection of common laboratory animals to assess the contribution of Borde-
tella virulence factors to pathogenesis. In addition to its postulated role as an adhesin (18), FHA appears to perform immunomodu-
laritory functions that contribute to colonization and/or persist-
tence (6, 12), though whether these effects are a direct result of some undefined FHA activity (19–23) or an indirect result of FHA-mediated adherence to specific host cells (6, 24, 25) is un-
clear.

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 (a general diagram of the domain structure is shown in Fig. S1 in the supplemental material). As with 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 (26), the signal peptide directs FhaB across the inner membrane via the Sec translocation machinery (27) and is then removed by leader pep-
tidase. The ~250-amino-acid (aa) region of FhaB immediately C terminal to the signal peptide, which is referred to as the TPS domain and which is highly conserved among TpsA proteins, is bound by chaperones that maintain the protein in a nonfolded state as it transits through the periplasm (28, 29). FhaB then tran-
sits through FhaC in an N- to C-terminal direction, with the N terminus remaining anchored to FhaC at the cell membrane (14, 26). The TPS domain initiates folding of FhaB into a rigid β-helix on the surface of the bacterium (30, 31), and progressive folding results in formation of an ~50-nm-long shaft. C terminal to the β-helical shaft, an ~500-aa globular domain begins to fold into the mature C-terminal domain (MCD) at the distal end of the mole-
cule (14, 32), which mediates adherence of the bacteria to host cells in vitro and to respiratory epithelium in vivo (13, 26, 28). Upon translocation of the residues composing the MCD, the proximal region of the FhaB prodomain (called the prodomain N terminus [PNT]), which is conserved among FhaB-like TpsA proteins, pro-
hits further translocation and retains the prodomain in an intracel-
ular compartment (26). 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 conforma-
tions that the MCD can sample during folding (26). 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 (14, 26), presumably due to misfolding of the MCD.

Subsequent to translocation and folding of the MCD, proteol-
ysis of the FhaB C-terminal prodomain occurs by the activity of an as-yet-unidentified protease(s) (14, 26). Degradation is rapid and complete (14, 26, 33), making it unlikely that the prodomain per-
forms an independent function. In ΔPNT strains, in which the prodomain is aberrantly translocated to the surface, the pro-
domain is readily detected (26), indicating that the prodomain is not intrinsically unstable but is instead subject to regulated degra-
dation inside the cell. Production of FhaB molecules lacking the C-terminal half of the MCD results in detectable, stable intracel-
ular prodomain polypeptides (26), further supporting the hy-
pothesis 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 (34). 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.

While adherence is the primary function attributed to FHA, our previous observations suggest that portions of the FhaB prodomain contribute to additional virulence activities mediated by FHA. For example, small deletions near the C terminus of the prodomain abrogated persistent tracheal infection of rats by B. bronchiseptica, though adherence capabilities in vitro were pre-
served (14). Here, we set out to determine the contribution of two C-terminal FhaB subdomains, a proline-rich region (PRR) and a conserved extreme C terminus (ECT), 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 (26). 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. Note that 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 (35). fhaS is a gene displaying high nucleotide sequence similarity to fhaB in B. bron-
chiseptica. However, deletion of fhaS does not produce any detect-
able effects on B. bronchiseptica pathogenicity in animal models (35), and we refer to RBX11 as the wild-type (WT) strain 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 (36). Western blot analysis of whole-cell lysates (WCL) and cul-
ture supernatants of the WT strain producing the fusion protein displayed no changes in the amount of FhaB or FHA (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 dot blot analysis of the strain producing FhaB with the C-terminal PhoA fusion, probing the blots with an α-MCD antibody (and with a secondary antibody with green flu-
orescence) and an α-PhoA antibody (and with a secondary anti-
body with red fluorescence). As seen previously (26), the MCD was detected on the surface of intact bacteria and in disrupted cells (Fig. 1b). In contrast, PhoA was detected only 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 fash-
ion (37), 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 degrada-
tion, while the PRR plays no apparent role in FhaB/FHA pro-
cessing. Western blot analysis of WT B. bronchiseptica revealed the
presence of ~370-kDa FhaB preproprotein and multiple processed FHA proteins of ~250 kDa in whole-cell lysates (WCL) and processed ~250-kDa FHA proteins only in culture supernatants (Sup.) from strains lacking the FhaB PRR (ΔPRR) or the FhaB ECT (ΔECT) or both (ΔPRR-ECT) or neither (WT), as well as each strain lacking sphB1, are shown. Strains lacking the FhaB ECT did not contain any observable FhaB in the WCL or Sup. fractions. Membranes were probed with α-MCD antibodies. The FhaB molecules translated by each strain are diagrammed at the top of the figure (dark blue, signal peptide; pink, TPS domain; purple, β-helical domain; green, MCD; brown, PNT; light blue, uncharacterized prodomain; red, PRR; yellow, ECT).

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

To investigate the contribution of the individual C-terminal subdomains to FhaB processing, we constructed B. bronchiseptica strains that produced FhaB proteins lacking either the PRR or the ECT. Similarly 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 a doublet that included FHA and a slightly smaller polypeptide that we had not observed previously but which was present also in culture supernatants of the WT strain (Fig. 2).
tion 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 (see Fig. S3 in the supplemental material) (26), suggesting that the reason for the strict conservation of the ECT in Bordetella cells 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. Additionally, stable prodomain fragments were not detected in WCL (see Fig. S2), indicating that prodomain removed from FhaB in the ΔECT strain is degraded rapidly, as it is in the parental strain.

Western blot analysis of WCL and culture supernatants of the ΔPRR strain, in 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 analysis. Similar to the WT strain results, the MCD was detected on the surface of intact bacteria and in disrupted cells and PhoA was detected only 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 ΔsphB1 strain (Fig. 2), as is seen with strains lacking the PNT (26). 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 nonciliated, nonpolarized cell lines and Bordetella cells adhere primarily to ciliated respiratory epithelium in vivo (39, 40), we developed an in vitro assay to assess the contribution of FHA to adherence to the respiratory tract (41). 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-negative (Bvg−) phase-locked strain that is completely nonadherent in vitro (Fig. 3) (42). Thus, ~30% recovery appears to be the upper limit of detection in this assay for nonadherent 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 2% 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, coinoculation of WT *B. bronchiseptica* with the ΔPRR strain, the ΔECT strain, or the FHA-null strain did not alter recovery of each 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* (14, 26). This mutation also resulted in aberrant folding of the FHA MCD (26). 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* (13). 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). Considered together 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 a 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 full-length 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 (14), even though this strain is capable of adhering both *in vivo* (Fig. 3) and *in vitro* (14). 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, 12, 13), WT bacteria persisted at high levels in the trachea and lungs through 11 days postinoculation, and FHA-null bacteria were mostly cleared from the trachea and lungs by 11 days postinoculation (Fig. 4a). The FHA-null strain displayed an increase in burden at 1 day postinoculation compared to WT bacteria, a bimodal distribution of burden at 3 days postinoculation, and a dramatic reduction in burden by day 11 (Fig. 4a). Additionally, as has been previously shown (2, 12–14), the number of CFU recovered from the nasal cavity for the FHA-null strain was similar to that for WT bacteria (see Fig. S4 in the supplemental material), indicating that FHA is not required for colonization and persistence in the upper respiratory tract of rodents.

Similarly 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 postinoculation (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 postinoculation and no bimodal burden distribution at 3 days postinoculation in the lungs (Fig. 4a). The ΔPRR strain also displayed increased clearance from the mouse trachea and lungs compared to WT bacteria (Fig. 4a), whereas, similarly to the results seen with the FHA-null bacteria, colonization and persistence in the nasal cavity were not compromised (see Fig. S4 in the supplemental material). 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 (see Fig. S5), demonstrating that the decreased persistence in the lower respiratory tract is due to the lack of the FhaB PRR rather than to the 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 the presence of 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, 12). 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 proinflammatory cytokines, such as interleukin-1β (IL-1β), and chemokines, such as the neutrophil chemoattractant KC and monocyte chemoattractant protein-1 (MCP-1), than WT bacteria (Fig. 4b). There was no difference in global levels of gamma interferon (IFN-γ), tumor necrosis factor α (TNF-α), IL-10, IL-12p70, IL-17, IL-22, or IL-23 produced in the lungs of mice infected with WT or FHA-null bacteria (see Fig. S6 in the supplemental material), 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 not due to a lack of FHA-mediated immunosuppression and that mature FHA is sufficient to suppress the initial inflammatory response.

**Full-length FhaB is required for resistance to early-immune-response-mediated clearance.** We previously demonstrated that coinoculation 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 the persistence of WT bacteria after coinoculation was unaltered compared to inoculation with only the WT strain (12). Infammation was suppressed after coinoculation compared to inoculation with only FHA-null bacteria; however, FHA-null bacteria were still not able to persist as well as WT bacteria (12), suggesting that FhaB/FHA may mediate resistance to clearance even under less-inflammatory conditions. Similarly to those previous findings, the number of CFU of WT bacteria recovered after coinoculation with the FHA-null strain was unchanged compared to the number recovered after inoculation with only WT bacteria, while the number of CFU of the FHA-null bacteria was increased compared to the number seen after inoculation with only FHA-null bacteria at 11 days postinoculation (Fig. 4 and 5a). Additionally, production of proinflammatory IL-1β and KC after coinoculation 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 postinoculation in the coinoculation 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 coinoculation of mice with WT and FHA-null bacteria, coinoculation 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 coinoculation 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 early 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 are highly regulated activities. The conserved N-terminal region of the prodomain is required for proper MCD folding and subsequent adherence capabilities (26). Additionally, we observed production of stable intracellular prodomain fragments in strains lacking the C-terminal portion of the MCD (26), 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 Fig. S7 in the supplemental material.

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 (41), 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 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 coinoculation 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 proinflammatory cytokines (IL-1β) and chemokines (KC, MCP-1) in the murine lung during coinfection. Cytokine and chemokine levels were determined from lung homogenates by ELISA. Data represent means ± SEM of the results determined for all samples, which were collected from all animals for which CFU are shown in Fig. 5a. Data are pooled from the results of 2 separate experiments conducted on different days. Statistical significance is indicated for mutant bacteria compared to the WT strain from the same coinoculation group. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
tein secretion, lar mechanisms underlying microbiological processes such as pro-
dermmber that, while culturing bacteria in the laboratory is ex-
der studies on the production or maturation of FHA or even from
ies on the production or maturation of FHA or even from

**MATERIALS AND METHODS**

**Bioinformatics.** Protein sequences were obtained from the NCBI Protein Database. Sequence alignments were conducted using Clustal Omega (47) and visualized using Jalview Version 2 (48). 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 (Col-

**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% trichlo-

**References**

1. Buscher AZ, Burmeister K, Barenkamp SJ, St Geme JW, III, 2004. Evolutionary and functional relationships among the nontypeable *Haemophilus influenzae* HMW family of adhesins. J Bacteriol 186:4209–4217. http://dx.doi.org/10.1128/JB.186.13.4209-4217.2004.

2. Cotter PA, Yuk MH, Mattow S, Akerley BJ, Boschwitz J, Relman DA, Miller JF. 1998. filamentous hemagglutinin of Bordetella bronchiseptica is required for efficient establishment of tracheal colonization. Infect Immun 66:5921–5929.

3. Melvin et al.
Dieterich C, Relman DA

21. McGuirk P, Mills KH. 2002. Pathogen-specific T regulatory cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of interleukin-17-mediated inflammation. Infect Immun 70:4023–4030.

22. McGuirk P, Mills KH. 2000. Direct anti-inflammatory effect of a bacterial virulence factor: IL-10-dependent suppression of IL-12 production by filamentous haemagglutinin from Bordetella pertussis. Eur J Immunol 30: 415–422. http://dx.doi.org/10.1002/1521-4141(20000230)2:4<145::AID -IMMU145>3.0.CO;2-X.

23. Dirix V, Mielcarek N, Debrue AS, Willery E, Alonso S, Versheure V, Mascart F, Locht C. 2014. Human dendritic cell maturation and cytokine secretion upon stimulation with Bordetella pertussis filamentous haemagglutinin. Microbes Infect 16:562–570. http://dx.doi.org/10.1111/j.1365-2958.2014.03107.x.

24. Gray MC, Donato GM, Jones FR, Kim T, Hewlett EL. 2004. Newly secreted adenylyl cyclase toxin is responsible for intoxication of target cells by Bordetella pertussis. Mol Microbiol 53:1709–1719. http://dx.doi.org/10.1111/j.1365-2958.2004.04227.x.

25. Zaretsky FR, Gray MC, Hewlett EL. 2002. Mechanism of association of adenylyl cyclase toxin with the surface of Bordetella pertussis: a role for toxin–filamentous haemagglutinin interaction. Mol Microbiol 45: 1589–1598. http://dx.doi.org/10.1046/j.1365-2958.2002.03107.x.

Noël CR, Mazar J, Melvin JA, Sexton JA, Cotter PA. 2012. The prodromal of the Bordetella two-partner secretion pathway protein FhaB remains intracellularly yet affects the conformation of the mature C-terminal domain. Mol Microbiol 86:688–1006. http://dx.doi.org/10.1111/mmi.12036.

Chevalier N, Moser M, Koch HG, Schimz KI, Willery E, Locht C, Jacob-Dubuissin F, Müller M. 2004. Membrane targeting of a bacterial virulence factor harbouring an extended signal peptide. J Mol Microbiol Biotechnol 8:7–18. http://dx.doi.org/10.1159/000080276.

Baud C, Hodak H, Willery E, Drobecq H, Locht C, Jamin M, Jacob-Dubuissin F. 2009. Role of DegP for two-partner secretion in Bordetella. Mol Microbiol 74:131–139. http://dx.doi.org/10.1111/j.1365-2958.2009.06860.x.

Hodak H, Wohlkönig A, Smet-Nonca C, Drobecq H, Wieruszski JM, Sénéchal M, Landrieu I, Locht C, Jamin M, Jacob-Dubuissin F. 2008. The peptidyl-prolyl isomerase and chaperone Par27 of Bordetella pertussis as the prototype for a new group of parulins. J Mol Biol 376:414–426. http://dx.doi.org/10.1016/j.jmb.2007.10.088.

Clamens C, Hodak H, Willery E, Locht C, Jacob-Dubuissin F, Villerot V. 2004. The crystal structure of filamentous haemagglutinin secretion domain and its implications for the two-partner secretion pathway. Proc Natl Acad Sci U S A 101:6194–6199. http://dx.doi.org/10.1073/pnas.0400291101.

Kajava AV, Cheng N, Cleaver R, Kessel M, Simon MN, Willery E, Jacob-Dubuissin F, Locht C, Steven AC. 2001. Beta-helix model for the filamentous haemagglutinin adhesin of Bordetella pertussis and related bacterial secretory proteins. Mol Microbiol 42:279–292. http://dx.doi.org/10.1046/j.1365-2958.2001.02598.x.

Makho AM, Hannah JH, Brennan MJ, Trus BL, Kocsis E, Conway JF, Wingfield PT, Simon MN, Steven AC. 1994. Filamentous haemagglutinin of Bordetella pertussis. A bacterial adhesin formed as a 50-nm monomeric rigid rod based on a 19-residue repeat motif rich in beta strands and turns. J Mol Biol 241:110–124. http://dx.doi.org/10.1006/jmb.1994.1478.

Delisse-Gathoye AM, Locht C, Jacob F, Raaschou-Nielsen M, Heron I, Ruelle JL, de Wilde M, Cabezón T. 1990. Cloning, partial sequence, expression, and antigenic analysis of the filamentous haemagglutinin gene of Bordetella pertussis. Infect Immun 58:2895–2005.

Coutte I, Antoine R, Drobecq H, Locht C, Jacob-Dubuisson F. 2001. Subtilisin-like autotransporter serves as maturation protease in a bacterial secretion pathway. EMBO J 20:5904–5908. http://dx.doi.org/10.1093/emboj/20.18.5040.

Julio SM, Cotter PA. 2005. Characterization of the filamentous haemagglutinin-like protein FhaA in Bordetella bronchiseptica. Infect Immun 73:4960–4971. http://dx.doi.org/10.1128/IAI.73.10.4960-4971.2005.

Hoffman CS, Wright A. 1985. Fusions of secreted proteins to alkaline phosphatase: an approach for studying protein secretion. Proc Natl Acad Sci U S A 82:5107–5111. http://dx.doi.org/10.1073/pnas.82.15.5107.

Mori H, Ito K. 2001. The Sec protein-translation pathway. Trends Microbiol 9:494–500. http://dx.doi.org/10.1016/S0966-842X(01)02174-6.

Locht C, Geoffroy MC, Renaud G. 1992. Common accessory genes for the Bordetella pertussis filamentous haemagglutinin and fimbriae share sequence similarities with the papC and papD gene families. EMBO J 11: 1275–1283.

Edwards JA, Groathouse NA, Boitano S. 2005. Bordetella bronchiseptica adherence to cilia is mediated by multiple adhesin factors and blocked by surfactant protein A. Infect Immun 73:3618–3626. http://dx.doi.org/10.1128/IAI.73.6.3618-3626.2005.

Paddock CD, Sanden GN, Cherry JD, Gal AA, Langston C, Tatti KM, Wu KH, Goldsmith CS, Greer PW, Montague JL, Elisson MT, Holman
41. Scheller EV, Melvin JA, Sheets AJ, Cotter PA. 2015. Cooperative roles for fimbria and filamentous hemagglutinin in *Bordetella* adherence and immune modulation. *mBio* 6:e00500-15. http://dx.doi.org/10.1128/mBio.00500-15.

42. Akerley BJ, Cotter PA, Miller JF. 1995. Ectopic expression of the flagellar regulon alters development of the *Bordetella*-host interaction. Cell 80: 611–620. http://dx.doi.org/10.1016/0092-8674(95)90515-4.

43. Adzhubei AA, Sternberg MJ, Makarov AA. 2013. Polyproline-II helix in proteins: structure and function. *J Mol Biol* 425:2100–2132. http://dx.doi.org/10.1016/j.jmb.2013.03.018.

44. Mobberley-Schuman PS, Weiss AA. 2005. Influence of CR3 (CD11b/CD18) expression on phagocytosis of *Bordetella pertussis* by human neutrophils. *Infect Immun* 73:7317–7323. http://dx.doi.org/10.1128/IAI.73.11.7317-7323.2005.

45. Weingart CL, Keitel WA, Edwards KM, Weiss AA. 2000. Characterization of bactericidal immune responses following vaccination with acellular pertussis vaccines in adults. *Infect Immun* 68:7175–7179. http://dx.doi.org/10.1128/IAI.68.12.7175-7179.2000.

46. Weingart CL, Weiss AA. 2000. *Bordetella pertussis* virulence factors affect phagocytosis by human neutrophils. *Infect Immun* 68:1735–1739. http://dx.doi.org/10.1128/IAI.68.3.1735-1739.2000.

47. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, Thompson JD, Higgins DG. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol* 7:539. http://dx.doi.org/10.1038/msb.2011.75.

48. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. 2009. Jalview, version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25:1189–1191. http://dx.doi.org/10.1093/bioinformatics/btp033.