

Cooperative Roles for Fimbria and Filamentous Hemagglutinin in *Bordetella* Adherence and Immune Modulation

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ABSTRACT *Bordetella fimbriae* (FIM) are generally considered to function as adhesins despite a lack of experimental evidence supporting this conclusion for *Bordetella pertussis* and evidence against a requirement for FIM in adherence of *Bordetella bronchiseptica* to mammalian cell lines. Using *B. bronchiseptica* and mice, we developed an *in vivo* adherence assay that revealed that FIM do function as critically important adhesins in the lower respiratory tract. In the first few days postinoculation, FIM-deficient *B. bronchiseptica* induced a more robust inflammatory response than wild-type bacteria did, suggesting that FIM, like filamentous hemagglutinin (FHA), allow *B. bronchiseptica* to suppress the innate immune response to infection. Localization analyses indicated that FIM are required for efficient attachment to airway epithelium, as bacteria lacking FIM localized to alveoli. FHA-deficient bacteria, in contrast, localized to airways. Bacteria unable to produce both FIM and FHA localized to alveoli and caused increased inflammation and histopathology identical to that caused by FIM-deficient bacteria, demonstrating that lack of FIM is epistatic to lack of FHA. Coinoculation experiments provided evidence that wild-type *B. bronchiseptica* suppresses inflammation locally within the respiratory tract and that both FHA and FIM are required for defense against clearance by the innate immune system. Altogether, our data suggest that FIM-mediated adherence to airway epithelium is a critical first step in *Bordetella* infection that allows FHA-dependent interactions to mediate tight adherence, suppression of inflammation, and resistance to inflammatory cell-mediated clearance. Our results suggest that mucosal antibodies capable of blocking FIM-mediated interactions could prevent bacterial colonization of the lower respiratory tract.

IMPORTANCE Although fimbriae (FIM) 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* FIM function as adhesins *in vivo*, specifically to airway epithelium. Furthermore, our results suggest that FIM mediate initial interactions with airway epithelial cells that are followed by tight filamentous hemagglutinin (FHA)-mediated binding and that together, FIM and FHA allow *Bordetella* to suppress inflammation, leading to prolonged colonization. Given the shortcoming of the current acellular component pertussis (aP) vaccine in preventing colonization, these findings suggest that generation of antibodies capable of blocking FIM-mediated adherence could potentially prevent *Bordetella* colonization.

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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 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 (aP) vaccines (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 viru-

lence factor is a type I pilus system, typically called fimbria (FIM) 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 FIM composed of either Fim2 or Fim3 as the major fimbrial subunit (10). The structural genes *fim2* and *fim3* are not linked to each other or to 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 sequences of the FimD proteins produced by *B. pertussis* (Tohama I) and *B. bronchiseptica* (RB50) are 95% identical, and the major fimbrial subunits, Fim2 and Fim3, are 73% and 94% identical, respectively (9, 17, 18). It is likely that the fimbriae 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 fimbriae have been shown to be important mediators of adherence for many bacterial pathogens, there is surprisingly little experimental evidence supporting this role for *Bordetella* FIM. 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 FIM 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 wild-type (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 FIM may be important for adherence specifically to ciliated respiratory epithelial cells. Although studies have been conducted to identify host cell receptors for FIM (23–25), these experiments used purified fimbrial subunits and nonciliated cell lines and whether the interactions identified reflect those that occur with native FIM *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 FIM 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 they colonized and persisted in the nasal cavity (20). These data suggest that FIM 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 per-

sistence in the lower respiratory tract and that FHA-deficient bacteria induce a more robust inflammatory response than WT bacteria do (27–29). These data suggest that FHA is involved in suppressing the host immune response to aid bacterial persistence. On the basis of the similar tracheal colonization defect of Δ *fhaB* and Δ *fimBCD* mutants in rats, we hypothesized that FIM may also contribute to immune modulation and bacterial persistence in the lower respiratory tract, and we set out to test this hypothesis.

RESULTS

Fimbriae are required for adherence *in vivo*. To investigate the contribution of FIM to adherence to respiratory epithelium in the context of natural infection, we developed an *in vivo* adherence assay. We inoculated mice intranasally with 50 μ l phosphate-buffered saline (PBS) containing 7.5×10^4 CFU of bacteria, euthanized the mice 30 min later, cannulated the tracheas, performed bronchoalveolar lavage (BAL) with 1 ml PBS, and determined the number of CFU recovered. When BAL was not performed, we recovered equivalent numbers of CFU for all strains tested, approximately 3.0×10^4 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 7.5×10^2 CFU in the BAL fluid (BALF) (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 postlavage lungs and recovered approximately 5×10^4 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 3×10^4 CFU in the lavage fluid, corresponding to ~55% of the recoverable CFU, while we recovered 7×10^3 CFU in the post-BAL lung homogenate, corresponding to ~15% of the recoverable CFU (Fig. 1A and B). 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 FIM (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 are (Fig. 1A and B). We conclude from these results that FIM contribute substantially to adherence to mouse respiratory tissue. Moreover, as FIM-deficient *B. bronchiseptica* was 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 $\sim 3 \times 10^4$ CFU in BALF from mice inoculated with either Δ *fhaB* or Δ *fimBCD* Δ *fhaB* bacteria, which is nearly identical to the number

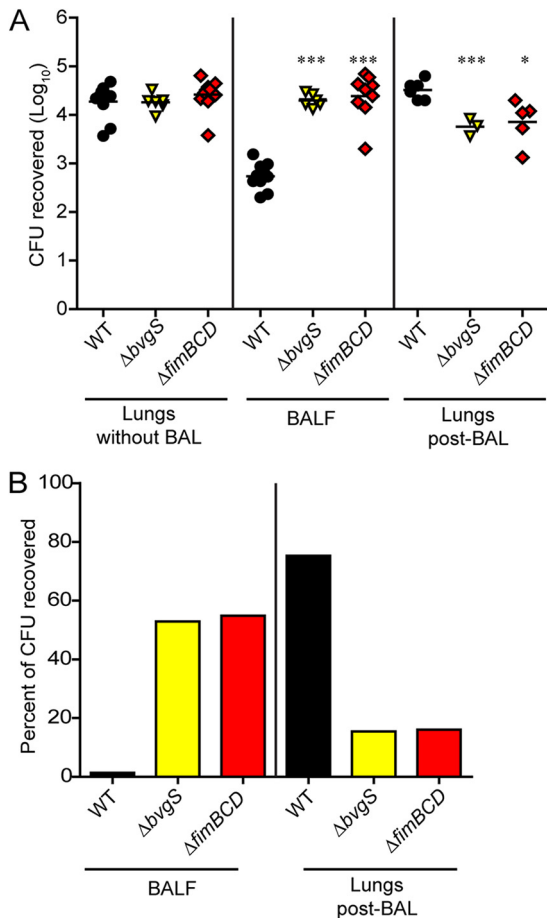


FIG 1 (A) Mice were inoculated intranasally with approximately 7.5×10^4 CFU of WT or mutant bacteria. The numbers of CFU recovered from lungs for which bronchoalveolar lavage (BAL) was not performed, from BAL fluid (BALF), and from lungs after BAL (post-BAL) are shown. Each symbol represents the value for an individual mouse, and the black bar shows the mean for the group of mice. Mean values for mutant bacteria that are significantly different from the mean value for WT bacteria are indicated by asterisks as follows: *, $P < 0.05$; ***, $P < 0.001$. (B) Data shown as the percentage of the recoverable CFU, which is calculated as the mean number of CFU recovered from either BALF or lungs post-BAL divided by the mean number of CFU recovered from lungs for which BAL was not performed for each strain. Values are means for two independent experiments.

recovered from FIM-deficient bacteria. In contrast, Δprn 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 $\Delta fimBCD$ and $\Delta fhaB$ mutant bacteria recovered from BALF was similar to that of the $\Delta bvgS$ 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.

Fimbriae are required for persistence in the lower respiratory tract. We inoculated 6-week-old BALB/c mice intranasally with 7.5×10^4 CFU of either wild-type, $\Delta fimBCD$, or $\Delta fimBCD$

$\Delta fhaB$ *B. bronchiseptica*. For mice inoculated with WT or $\Delta fimBCD$ mutant bacteria, we determined bacterial burden in the nasal cavity, trachea, and right lung lobes at various times postinoculation (p.i.). For mice inoculated with the $\Delta fimBCD$ $\Delta fhaB$ mutant, we determined bacterial burden only in the lungs. CFU recovered from tissues harvested 1 h p.i. (day 0) were similar among all animals, indicating that consistent inoculation between bacterial strains and replicates occurred (Fig. 2A to C). There was no difference in the number of CFU recovered from the nasal cavities of animals inoculated with WT or $\Delta fimBCD$ bacteria at any time p.i. (Fig. 2A). We also recovered similar numbers of CFU of WT and $\Delta fimBCD$ bacteria from the tracheas 1 and 3 days p.i. However, 14 days p.i., no $\Delta fimBCD$ 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, fimbriae are required for persistence in the tracheas of mice.

We recovered approximately 1-log-unit-more CFU of $\Delta fimBCD$ or $\Delta fimBCD$ $\Delta fhaB$ bacteria than WT bacteria from the lungs 1 day p.i. (Fig. 2C). At 3 days p.i., mice inoculated with $\Delta fimBCD$ 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 bacterial burden of the second group was similar to the burden of mice inoculated with WT bacteria, and these mice showed no signs of respiratory distress. This “bimodal” phenotype at 3 days p.i. is similar to what has been observed in mice inoculated with $\Delta fhaB$ bacteria (27–29). For mice inoculated with $\Delta fimBCD$ $\Delta fhaB$ mutants, the bacterial burden was not clearly bimodal but spread between 10^5 and 10^7 bacteria at 3 days p.i. By 7 days p.i., the burdens of both the $\Delta fimBCD$ bacteria and $\Delta fimBCD$ $\Delta fhaB$ bacteria were significantly lower than the burden of WT bacteria, and the mutants were undetectable by 14 days p.i.

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

Fimbriae 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 alveoli during infection (Fig. 3A). The lungs of mice inoculated with only PBS appeared healthy, with little, if any, evidence of inflammation at any time point p.i. The lungs of mice inoculated with WT bacteria contained cellular infiltrates around the major airways (Fig. 3A, black arrows), but the alveoli and alveolar spaces were free of any signs of inflammation at 3 days p.i. The cellular infiltrates around major airways persisted to 7 days p.i. in mice inoculated with WT bacteria (Fig. 3A, black arrow), coinciding with the high bacterial burden at this time point. The lungs of mice inoculated with $\Delta fimBCD$ bacteria displayed some cellular infiltration around the major airways but also showed substantial visually distinct cellular infiltrate within the alveolar spaces at 3 days p.i. (Fig. 3A, 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 7 days p.i., the patches of inflammatory cell recruitment

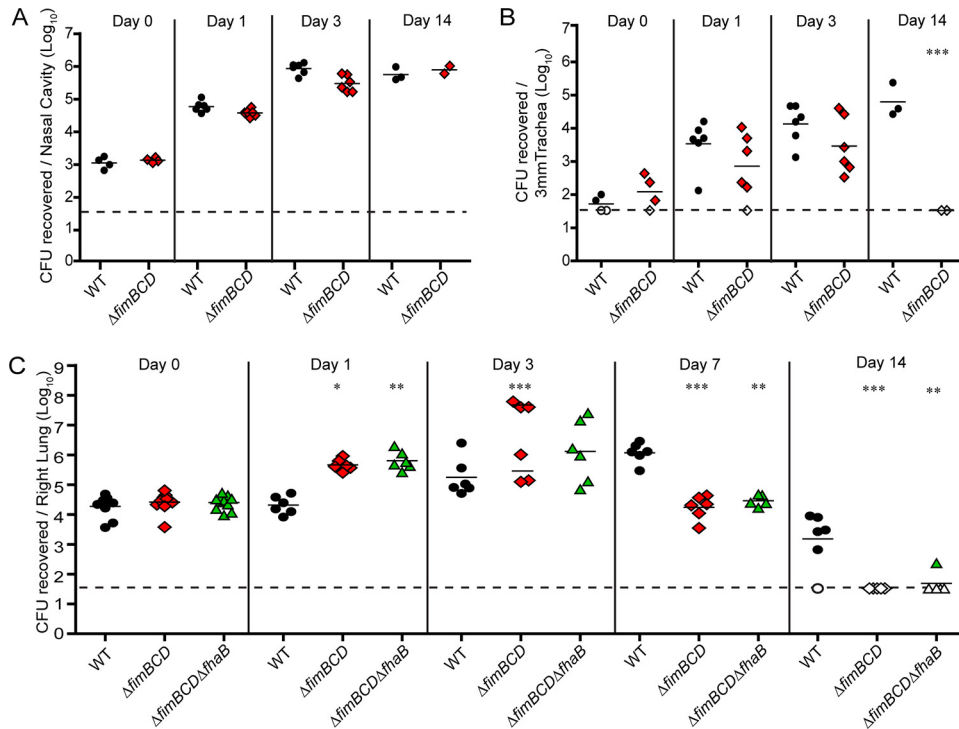


FIG 2 (A to C) Bacterial burden in respiratory tissues from mice inoculated with WT or mutant bacteria. Each symbol represents the value for an individual animal, and the black bar represents the mean for the group. The horizontal dashed line represents the lower limit of detection. Values are means 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: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

in alveoli of $\Delta fimBCD$ mutant-inoculated mice was absent, and there was decreased cellular infiltrate around the major airways, corresponding with the decreased bacterial burden at this time point. The lungs of mice inoculated with $\Delta fimBCD \Delta fhaB$ bacteria appeared similar to those of mice inoculated with $\Delta fimBCD$ 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 (Fig. 3A, black arrows) at 3 days p.i. At 7 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 in enzyme-linked immunosorbent assays (ELISAs) (Fig. 3B). Interleukin-1 β (IL-1 β) levels were significantly increased in the lungs of mice inoculated with $\Delta fimBCD$ or $\Delta fimBCD \Delta fhaB$ bacteria compared to WT bacteria 1 and 3 days p.i. These differences did not correlate with bacterial burden, as IL-1 β levels were increased in all animals inoculated with the mutant bacteria, even those with lower bacterial burdens at day 3 p.i. Moreover, monocyte chemotactic protein 1 (MCP-1) and neutrophil chemokine CXCL1 (CXC chemokine ligand 1) (KC [keratinocyte-derived chemokine]) levels were significantly increased in the lungs of mice inoculated with $\Delta fimBCD$ bacteria compared to the lungs of mice inoculated with WT bacteria 3 days p.i. MCP-1 and KC levels in mice inoculated with $\Delta fimBCD \Delta fhaB$ mutant bacteria were higher than in mice inoculated with WT bacteria, but the differences were not statistically significant. Other cytokines, such as interferon gamma (IFN- γ), tumor necrosis factor alpha (TNF- α), interleukin-10, -12p70, -22, and -23 (IL-10, IL-12p70, IL-22, and IL-23) were measured, and no signif-

icant difference was found between mice inoculated with $\Delta fimBCD$ or $\Delta fimBCD \Delta fhaB$ bacteria compared to those inoculated with WT bacteria at any time point.

Taken together, these data indicate that FIM, 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 $\Delta fimBCD \Delta fhaB$ double mutant induced a histopathology pattern similar to that induced by the $\Delta fimBCD$ mutant indicates that the $\Delta fimBCD$ mutation is epistatic to the $\Delta fhaB$ mutation.

Fimbriae do not complement “in trans” during coinoculation. 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 coinoculated with WT and FHA-deficient bacteria, the level of inflammation in the lungs is less than that induced by inoculation with the $\Delta fhaB$ mutant alone, and the $\Delta fhaB$ 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 1.5×10^5 CFU of WT, $\Delta fimBCD$, or $\Delta fimBCD \Delta fhaB$ bacteria alone or a mixture of 7.5×10^4 CFU each of WT and $\Delta fimBCD$ bacteria or WT and $\Delta fimBCD \Delta fhaB$ bacteria (so that the total number of CFU in each inoculum was 1.5×10^5) and determined the bacterial burden.

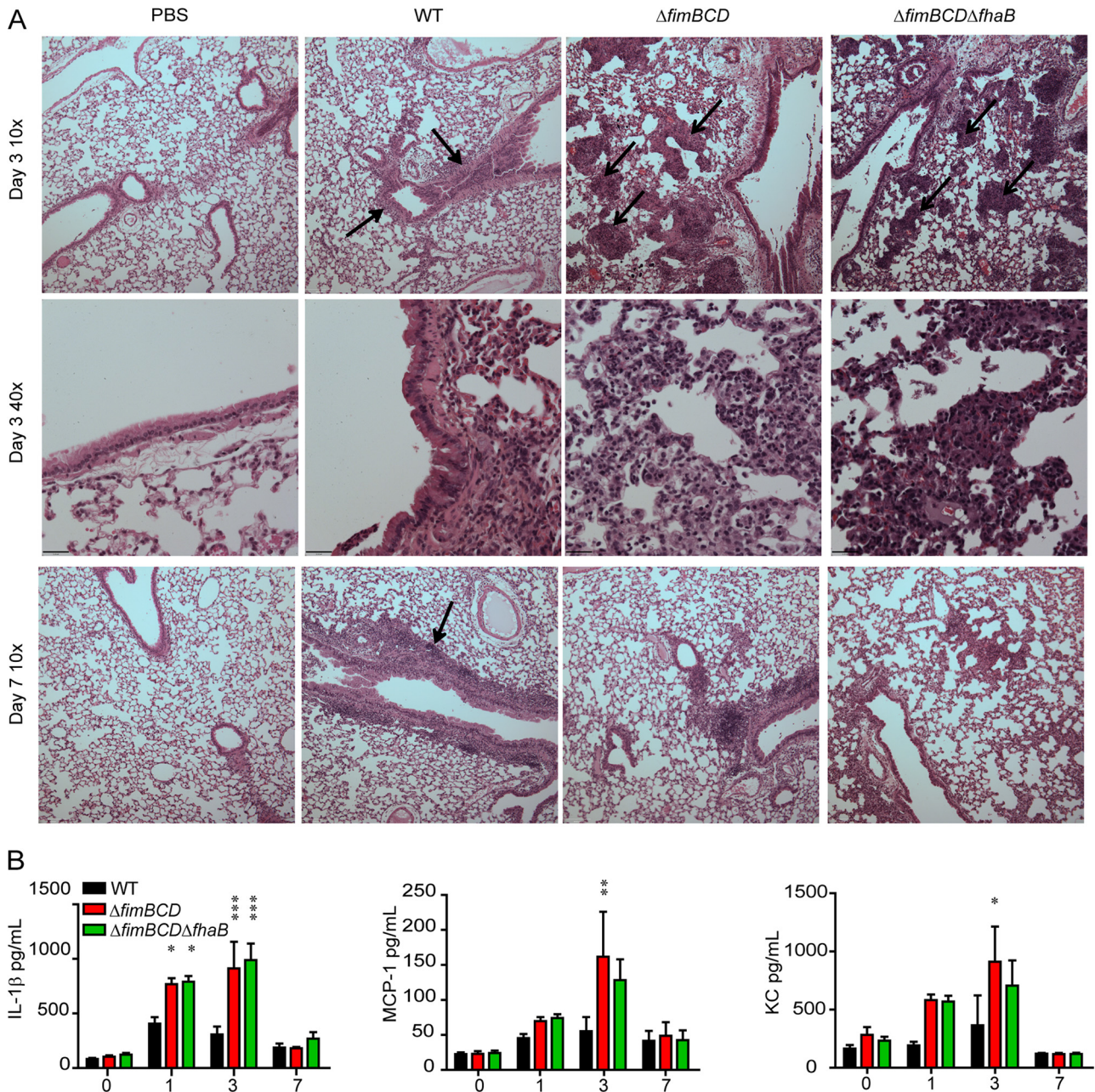


FIG 3 (A) Hematoxylin-and-eosin-stained 5- μ m lung sections at $\times 10$ and $\times 40$ magnification. Black arrows indicate areas of cellular infiltrate. (B) Cytokine and chemokine levels in lung homogenates of animals inoculated with WT bacteria or with $\Delta fimBCD$ or $\Delta fimBCD \Delta fhaB$ mutant bacteria. The y axis shows time postinoculation (in days). Values are means \pm standard errors (SE) (error bars) 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: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Strikingly, the numbers of mutant and WT bacteria recovered from the trachea and lungs from coinoculated animals were similar to those 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 $\Delta fimBCD$ or $\Delta fimBCD \Delta fhaB$ bacteria in *trans*, further supporting the hypothesis that FIM and FHA contribute differently to infection.

Lung sections from mice coinoculated with WT and either mutant strain appeared similar to lung sections of mice inoculated with $\Delta fimBCD$ or $\Delta fimBCD \Delta fhaB$ bacteria alone: there was cell recruitment around the major airways as well as in the alveolar spaces (Fig. 4B, 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-1 β , KC, and MCP-1 were higher in coinoculated mice than in mice inoculated with WT bacteria alone, although most differences were not statistically significant (Fig. 4C).

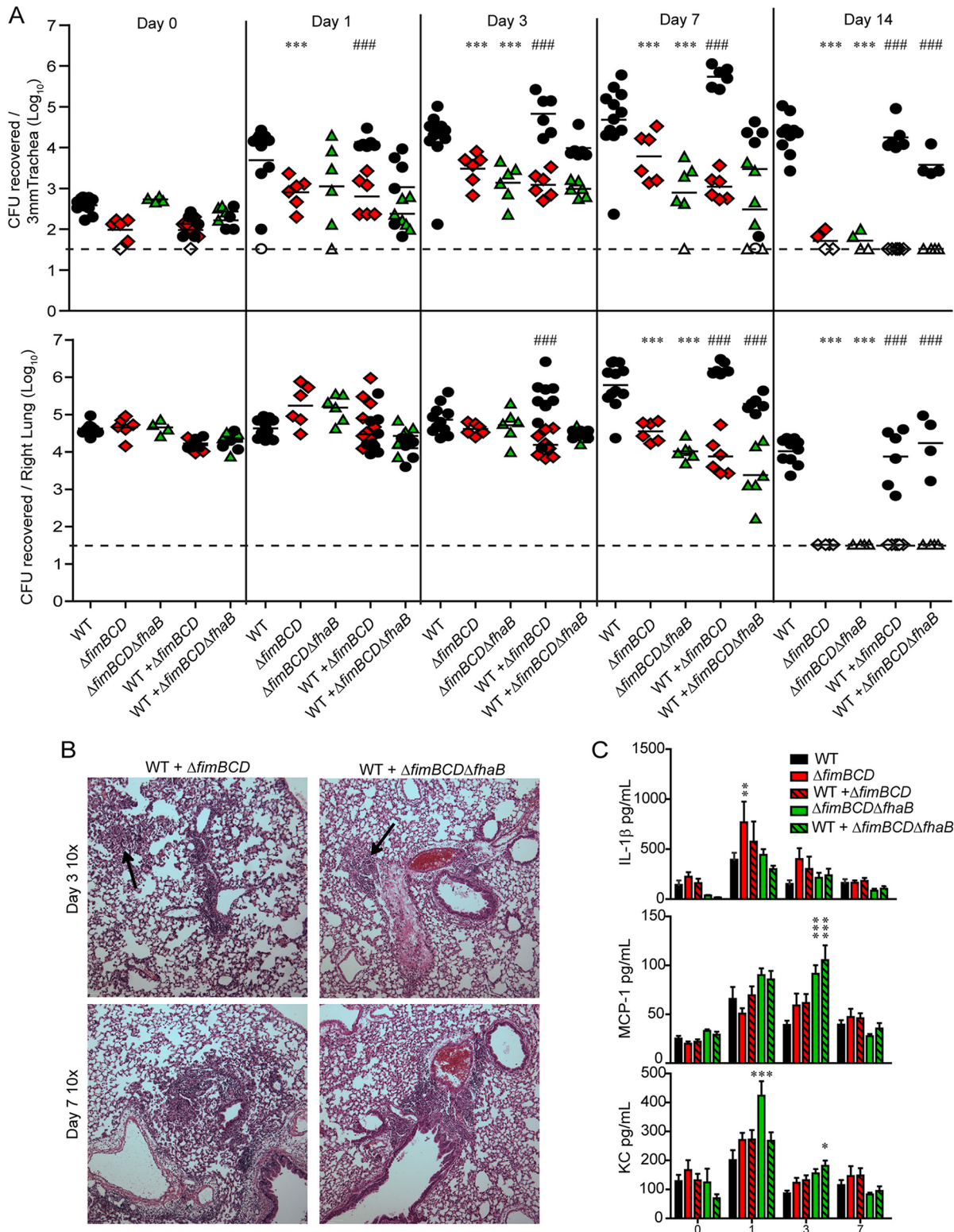


FIG 4 (A) Bacterial burden in respiratory tissue from mice inoculated with WT or mutant bacteria alone or coinoculated with WT and mutant bacteria. Each symbol represents the value for an individual animal, and the black bar represents the mean of the group of animals. The horizontal dashed line represents the lower limit of detection. (B) Hematoxylin-and-eosin-stained 5- μ m lung sections at a magnification of $\times 10$. Arrows indicate areas of cellular infiltration. (C) Cytokine and chemokine levels in lung homogenates of animals inoculated with WT bacteria or $\Delta fimBCD$ or $\Delta fimBCD \Delta fhaB$ mutant bacteria. Values are means (A) or means \pm SE (C) for at least two independent experiments. Mean values for single mutant strains that are significantly different from the mean value for WT bacteria are indicated by asterisks as follows: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. Mean values for each strain in coinoculated animals that are significantly different from each other are indicated (###).

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 from entering alveolar space. 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 7.5×10^4 CFU of WT or mutant bacteria and then sacrificed the mice 3 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. The lungs of mice inoculated with WT bacteria showed red staining around the ciliated epithelium of the major airways (Fig. 5, black arrows), while control lungs, which were not incubated with rabbit serum, did not have any red staining (Fig. 5). This staining pattern indicates that WT bacteria localize to the major airways during infection.

The lungs of mice inoculated with FHA-deficient bacteria appeared similar to the lungs of mice inoculated with WT bacteria, with noticeable red staining around the major airways, both on the ciliated epithelium and in the cellular infiltrate beneath the epithelial cells (Fig. 5A, black arrows), but very little red staining in the alveolar space, suggesting that FHA-deficient bacteria localize similarly to WT bacteria. In contrast, the lungs of mice inoculated with FIM-deficient bacteria showed a dramatically different staining pattern. These lungs had red staining throughout the alveolar space (Fig. 5A, black arrows). The lungs of mice inoculated with $\Delta fimBCD \Delta fhaB$ double mutant bacteria appeared similar to the lungs of 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 FIM, many, if not most, bacteria bypass the ciliated epithelium and are deposited in alveoli.

The lungs of mice coinoculated with WT and FHA-deficient bacteria had red staining around the major airways (Fig. 5B, black arrows), but very little red staining in alveolar space, similar to the lungs of mice inoculated with WT or FHA-deficient bacteria alone. In contrast, the lungs of mice coinoculated with WT and either $\Delta fimBCD$ or $\Delta fimBCD \Delta fhaB$ bacteria had red staining in the major airways and distinct staining in alveolar space (Fig. 5B, black arrows). These data suggest that even during coinoculation, 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 coinoculation with WT bacteria can rescue FHA-deficient bacteria but not FIM-deficient bacteria: immune suppression mediated by WT bacteria occurs locally in the major airways and does not affect bacteria in 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 inoc-

ulated 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 FIM are generally considered to function as adhesins despite there being no reports of adherence studies using *B. pertussis* strains defective only for production of FIM. Studies of *B. bronchiseptica* also failed to provide convincing evidence that *Bordetella* FIM function as adhesins, as FIM-deficient bacteria showed no defect in adherence assays using a variety of nonciliated 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* FIM are important adhesins, and they indicate that fimbriae mediate adherence specifically to airway epithelium. Our results also show that FIM 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, predominantly neutrophils, to the site of infection. In this model, both bacterial burden and cellular infiltrate peak at about 7 days postinoculation. Bacterial load then decreases, with clearance from the lungs occurring over the next 2 to 3 weeks and requiring adaptive immunity (16). For *B. pertussis*, the bacteria are cleared from the entire respiratory tract by about 30 to 40 days postinoculation, while for *B. bronchiseptica*, the bacteria are cleared from the 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* bacteria are not distributed evenly throughout the lungs following high-dose, large-volume inoculation. Instead they localize predominantly to airway epithelium. This localization requires FIM, 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 FIM-mediated interactions that induce changes in either bacterial or host cells. FHA-deficient bacteria, in contrast, localized to the airways, presumably due to fimbrial attachment to ciliated epithelium, but these FIM-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 FIM and FHA. In this model, fimbriae 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 3 or 4 days postin-

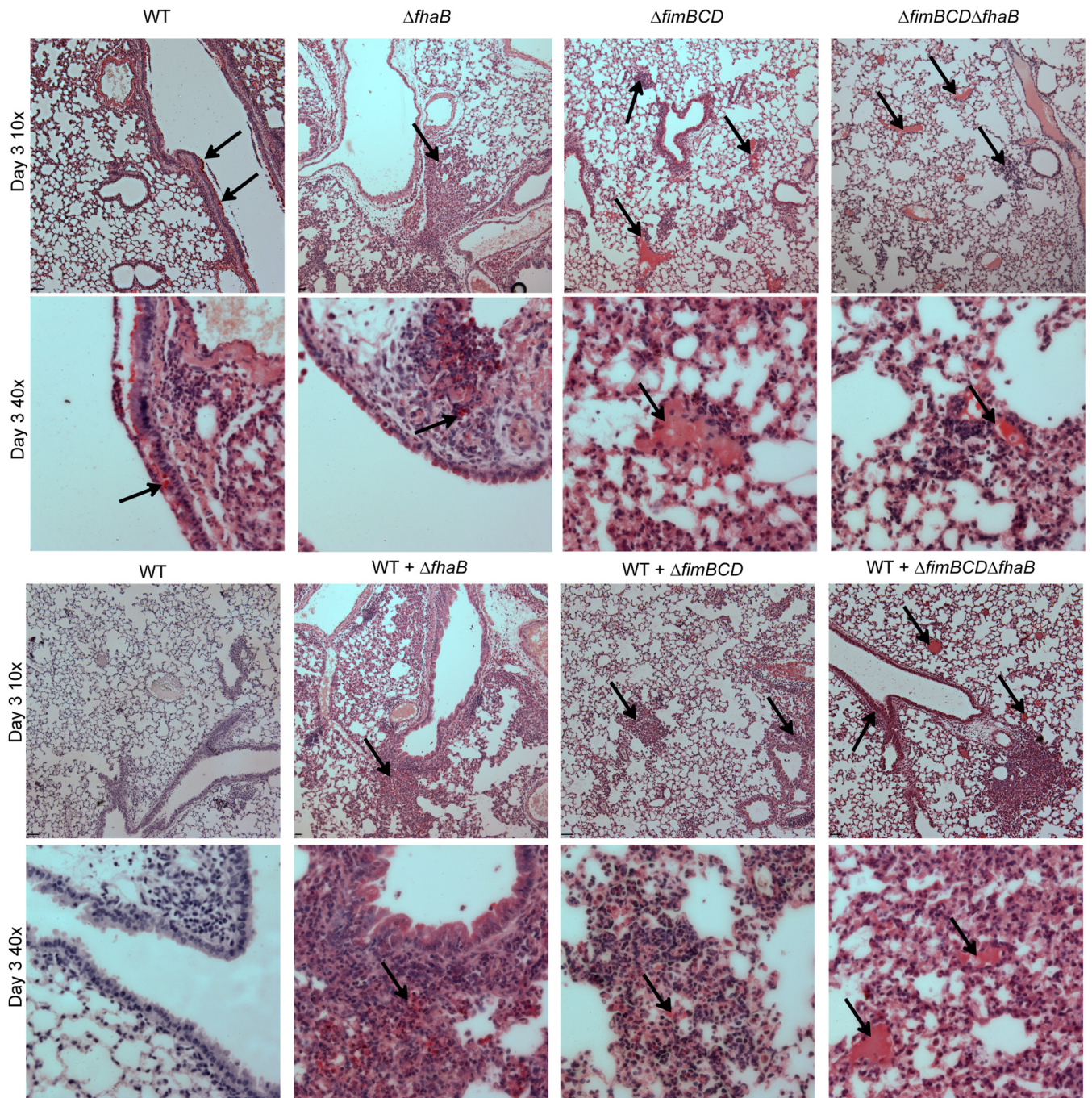


FIG 5 Lung sections 3 days p.i. from mice inoculated with WT or mutant bacteria (A) or coinoculated with WT and mutant bacteria (B). Immunohistochemistry was performed using convalescent rabbit serum from a rabbit infected with WT bacteria and goat anti-rabbit secondary antibody conjugated to alkaline phosphatase, incubated with naphthol red substrate. Red staining (black arrows) indicate alkaline phosphatase activity. Tissue was counterstained with hematoxylin. Pictures were taken at magnifications of $\times 10$ and $\times 40$. Control indicates no primary antibody staining.

oculation. We have previously shown a role for FHA in modulating the innate immune response, as FHA-deficient *B. bronchiseptica* bacteria were hyperinflammatory 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 proinflammatory cytokines and chemokines during the first 3 days of infection, suggesting that FIM also contribute to

suppression of inflammation (Fig. 2). The lungs of 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 localizations 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 $\Delta fimBCD \Delta fhaB$ bacteria were similar to those of mice inoculated with $\Delta fimBCD$ bacteria. These data indicate that the lack of FIM is epistatic to the lack of FHA; without FIM, the presence or absence of FHA did not change the outcome of infection, underscoring the importance of FIM for FHA-mediated interactions.

In this murine model, bacterial burden and inflammatory infiltrate in the lungs both peak at about 7 days postinoculation. 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 hyperinflammatory 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 coinoculated with WT and FHA-deficient bacteria exhibit less inflammation than mice inoculated with FHA-deficient bacteria alone. Furthermore, in coinoculated animals, there was increased persistence of FHA-deficient bacteria than in animals inoculated with only the $\Delta fhaB$ mutant (29). We hypothesized that the increased persistence of $\Delta fhaB$ bacteria in coinoculated animals resulted from FHA-producing WT bacteria suppressing inflammation. We also hypothesized that the decreased ability of FHA-deficient mutants compared to WT bacteria to persist in coinoculated animals indicated a decreased ability of FHA-deficient bacteria to resist phagocytic cells. Our new results show that WT and FHA-deficient bacteria colocalize 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.

In contrast, WT bacteria did not improve the survival of FIM-deficient bacteria, which were cleared as rapidly from mice coinoculated with WT bacteria as from mice inoculated with FIM-deficient bacteria alone (Fig. 3). The inability of WT bacteria to complement “in trans” the $\Delta fimBCD$ mutant (and the $\Delta fimBCD \Delta fhaB$ 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 $\Delta fimBCD$ and $\Delta fimBCD \Delta fhaB$ 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 FIM are required for lower respiratory tract colonization of these hosts by *B. bronchiseptica*. However, *B. bronchiseptica* in-

fection 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 in *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 whether the genes encoding FIM in *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 FIM was defective for persistence and caused increased cellular infiltrate in the alveoli of mice, a study in which the authors concluded that FIM were important for modulating the immune response (35). We hypothesize, therefore, that *B. pertussis* FIM are required for adherence to airway epithelium and for FHA-mediated immunomodulation during human infection. Testing this hypothesis and determining whether 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 are 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 National Institutes of Health (42). Our protocol was 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. *Escherichia coli* was 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) agar again (Becton Dickinson Microbiology Systems) supplemented with 7.5% defibrinated sheep blood (Colorado Serum Co., Denver, CO) or in Stainer-Scholte (SS) broth with 100 mg/ml (2,6-*O*-dimethyl)- β -cyclodextrin. When necessary, media were supplemented with streptomycin (Sm) (20 μ g/ml), gentamicin (Gm) (30 μ g/ml), kanamycin (50 μ g/ml), or diaminopimelic acid (DAP) (300 μ g/ml).

Construction and cloning of plasmids were accomplished in *E. coli* DH5 α . 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

TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description	Reference
<i>E. coli</i> strains		
DH5 α	Molecular cloning strain	30
RH03	Conjugation strain; Km ^r ; DAP auxotroph	39
<i>B. bronchiseptica</i> strains		
RB50	“Wild-type” <i>Bordetella bronchiseptica</i> complex I strain	14
RB50 Km ^r	RB50 containing constitutively expressed <i>nptII</i> inserted via a pUC18-based plasmid at the <i>attTn7</i> site	This study
RB63	RB50 containing deletion of the <i>fimBCD</i> operon	20
RB63 Gm ^r	RB63 containing constitutively expressed <i>aacC1</i> inserted via a pUC18-based plasmid at the <i>attTn7</i> site	This study
SP5	RB50 containing an in-frame deletion of codons 227 to 756 of <i>prn</i>	22
RBX9f	RBX9 with a deletion mutation of the <i>fimA-fhaB</i> intergenic region	31
RB515	RB50 containing a deletion in <i>cyaA</i>	27
WD3	RB50 with an in-frame deletion of <i>bscN</i>	40
RB54	RB50 Bvg ⁻ phase-locked variant with an in-frame deletion of <i>bvgS</i>	14
AS16	RB63 with a mutation of the <i>fhaB</i> -MCD	This study
AS16 Gm ^r	AS16 containing constitutively expressed <i>aacC1</i> inserted via a pUC18-based plasmid at the <i>attTn7</i> site	This study
Plasmids		
pSS4245	pBR322-based allelic exchange plasmid; Ap ^r Km ^r	30
pTsS3	Tn7 transposase expression vector containing <i>tnsABCD</i> ; Ap ^r	41
pUC18-miniTn7	Transposition vector; Ap ^r Km ^r	41

resistance (*nptII*) to the *attTn7* site via transposase-mediated insertion. Detailed descriptions of each strain are given in Table 1.

Intranasal mouse inoculation. Bacteria were grown overnight in Stainer-Scholte broth with 100 mg/ml (2,6-*O*-dimethyl)- β -cyclodextrin. When necessary, media were supplemented with streptomycin (20 μ g/ml), gentamicin (30 μ g/ml), or kanamycin (50 μ g/ml). Six-week-old BALB/c mice from Jackson Laboratories (Bar Harbor, ME) were inoculated intranasally with 7.5×10^4 or 1.5×10^5 CFU of *B. bronchiseptica* in 50 μ l PBS. Mice were inoculated with strains RB50, RB63, RBX9f, RB54, SP5, and AS16 or a mixture of strains RB50 and RB63 or strains RB50 and AS16. The right lung lobes, tracheae, and nasal cavities were harvested from mice at specific time points postinoculation (p.i.) in 1 ml PBS. Tissue was homogenized, serial dilutions were plated on BG agar, and the number of CFU were determined.

Cytokine and histological analyses. 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 IL-1 β , KC, MCP-1 and IL-17 cytokines and chemokines were measured following the manufacturer’s instructions. The cytokine 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 the time points indicated in the figures and inflated with 10% formalin. The Animal Histopathology Core Lab then embedded the tissue in paraffin, sectioned the tissue so that the tissue section was 5 μ m thick, and then stained the tissue with hematoxylin and eosin (H&E). Lung sections were examined at the Microscopy Services Laboratory using bright-field imaging on an Olympus BX61 microscope at magnifications of $\times 10$ and $\times 40$.

In vivo adherence. Bacteria were grown as described above, and 6-week-old BALB/c mice were inoculated with 7.5×10^4 CFU of bacteria. Thirty minutes p.i., the mice were euthanized, the tracheae were 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 the number of CFU recovered.

Bacterial localization. Six-week-old BALB/c mice were inoculated as described above. Lung tissue was harvested 3 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- μ m lung sections. The tissue sections were incubated with serum from a rabbit chronically infected with *B. bronchiseptica* RB50 bacteria and then incubated with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Promega). The tissue sections were then incubated with alkaline phosphatase substrate naphthol red (Sigma), counterstained with hematoxylin (Sigma), and examined on an Olympus Bx61 microscope at magnifications of $\times 10$ and $\times 40$.

Statistical analysis. Statistical analysis was 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.).

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