

Bordetella bronchiseptica Adherence to Cilia Is Mediated by Multiple Adhesin Factors and Blocked by Surfactant Protein A

Jessica A. Edwards,^{1,2} Nathan A. Groathouse,³ and Scott Boitano^{1,2,4*}

Department of Cell Biology and Anatomy,¹ Arizona Respiratory Center,² and Department of Physiology,⁴ University of Arizona Health Sciences Center, Tucson, Arizona 85724, and Department of Microbiology, Immunology and Pathology Colorado State University, Fort Collins, Colorado 80523-1682³

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In the virulent state (Bvg⁺), *Bordetella bronchiseptica* expresses adhesins and toxins that mediate adherence to the upper airway epithelium, an essential early step in pathogenesis. In this study, we used a rabbit tracheal epithelial cell binding assay to test how specific host or pathogen factors contribute to ciliary binding. The host antimicrobial agent surfactant protein A (SP-A) effectively reduced ciliary binding by Bvg⁺ *B. bronchiseptica*. To evaluate the relative contributions of bacterial adhesins and toxins to ciliary binding, we used mutant strains of *B. bronchiseptica* in the binding assay. When compared to Bvg⁺ or Bvg⁻ phase-locked *B. bronchiseptica* strains, single-knockout strains lacking one of the known adhesins (filamentous hemagglutinin, pertactin, or fimbriae) displayed an intermediate ciliary binding capacity throughout the coinoculation. A *B. bronchiseptica* strain deficient in adenylate cyclase-hemolysin toxin also displayed an intermediate level of adherence between Bvg⁺ and Bvg⁻ strains and had the lowest ciliary affinity of any of the Bvg⁺ phase strains tested. A *B. bronchiseptica* strain that was missing dermonecrotic toxin also displayed intermediate binding; however, this strain displayed ciliary binding significantly higher than most of the adhesin knockouts tested. Taken together, these findings suggest that virulent-state *B. bronchiseptica* expresses multiple adhesins with overlapping contributions to ciliary adherence and that host production of SP-A can provide innate immunity by blocking bacterial adherence to the ciliated epithelium.

Members of the genus *Bordetella* are gram-negative coccobacilli that can cause respiratory disease in humans and other mammals and include *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. *B. pertussis* is a strict human pathogen and is the causative agent of pertussis (whooping cough), a disease that remains endemic to adult populations despite extensive vaccination programs initiated in the 1940s (3). Subspecies of *B. parapertussis* cause a milder pertussis-like disease in humans (20) but have also been associated with nonhuman disease (36). *B. bronchiseptica* infects a wide range of four-legged mammals and results predominantly in asymptomatic infections, although cases of symptomatic disease such as atrophic rhinitis in swine, kennel cough in dogs, and snuffles in rabbits have been well documented (12). Despite their varied host range, recent sequencing of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* has shown a high conservation of the pathogenic machinery possibly derived from a single *B. bronchiseptica*-like ancestor (35). The three strains express highly similar virulent protein profiles under the control of the *Bordetella* virulence gene activator/sensor two-component signal transduction system (BvgAS) (51). Activation of the virulent (Bvg⁺) phase is required for successful colonization of the respiratory tract (4, 28, 32).

The conducting airway is protected by an epithelium that provides a physical barrier between respired air and the underlying tissue of the upper respiratory tract. The epithelium generates a “mucociliary escalator” to clear particulate mate-

rials, including pathogenic bacteria, from the airway and keep them from reaching the lower lungs (40). The innate immune function of the conducting airway is further complemented by secretion of several antimicrobial molecules that include collectins such as surfactant proteins A and D (SP-A, SP-D) (6). During successful colonization of the mammalian respiratory tract, *Bordetella* spp. overcome or evade these and other early innate immune defenses, in part by directly binding to the cilia of the respiratory epithelium (33, 45).

Following access of the bacteria to the upper airway, *Bordetella* can mediate and maintain ciliary adherence via redundant and/or sequential interactions between bacterial attachment molecules and the host cell cilia. Such factors implicated in host-cell attachment and subsequent colonization include filamentous hemagglutinin (FHA), pertactin, fimbriae, and adenylate cyclase-hemolysin (CyaA) toxin (27). These molecules have been studied both in vitro and in vivo to assess their contribution as adhesins and their ability to contribute to colonization of the upper respiratory tract. FHA from *B. pertussis* has been shown to contribute to human ciliated cell binding (48, 49) and aciliated cell binding (31, 37, 50). FHA from *B. bronchiseptica* has been shown to mediate binding to L2 cells (43) and to be necessary, but not sufficient, for the colonization of rat trachea (5). Pertactin can contribute to the binding of *B. pertussis* to CHO (25) or HEp-2 (50) cell lines and has a crystal structure that shows a variety of candidate protein binding sites (10). Fimbriae can contribute to *B. pertussis* binding to monocytes (18, 19) and to HEp-2 cells (50). CyaA protein can directly bind to erythrocytes (13), and loss of *cyaA* from *B. pertussis* can reduce attachment to human ciliated cells (49). A *B. bronchiseptica* strain that does not express dermonecrotic toxin (DNT) has been shown to differentially colonize swine

* Corresponding author. Mailing address: Arizona Respiratory Center, Room 2338, AHSC Arizona Health Sciences Center, 1501 N. Campbell Avenue, Tucson, AZ 85724-5030. Phone: (520) 626-2105. Fax: (520) 626-6970. E-mail: sboitano@email.arizona.edu.

TABLE 1. *Bordetella bronchiseptica* strains used in this study^a

Strain	Genotype	Comments	Reference or source
RB50	WT ^b	Wild-type isolate from rabbit nares; can switch between Bvg ⁺ and Bvg ⁻ dependent on environmental conditions	4
RB53	<i>bvgS</i> -C3	RB50 derivative; a single base pair change in <i>bvgS</i> that results in a phase-locked Bvg ⁺ strain independent of growth conditions	4
RB54	Δ <i>bvgS</i>	RB50 derivative; a 1.4-kb deletion in <i>bvgS</i> that results in a phase-locked Bvg ⁻ strain independent of growth conditions	4
RB57	Δ <i>bvgS</i> , Δ <i>flaA</i>	RB54 derivative; Bvg ⁻ phase locked that additionally does not express flagella	1
DF8	Δ <i>bvgS</i> , Δ <i>flaA</i> , <i>fhaB</i> ^r , <i>fhaC</i> ^r	RB57 derivative; Bvg ⁻ phase locked, lacking flagella that ectopically expresses FHA	5
RBX9	Δ <i>fhaB</i>	RB50 derivative; does not express FHA	5
SP5	Δ <i>prn</i>	RB50 derivative; does not express pertactin	See Materials and Methods
RB63	Δ <i>fim</i>	RB50 derivative; does not express fimbrial adhesin	29
RB58	Δ <i>cyaA</i>	RB50 derivative; does not express CyaA toxin	16
50 Δ <i>dnt</i>	Δ <i>dnt</i>	RB50 derivative; does not express DNT	This study

^a All strains used were cultured in Bvg⁺ phase conditions.

^b WT, wild type.

airways (2). DNT has been suggested to alter ciliary binding in *B. avium* (47) and may contribute to *B. bronchiseptica* pathogenesis through host cell adhesion.

Despite evidence for each of the proteins above to contribute to adhesion, many of the studies discussed above have focused on binding to aciliated cells or ciliated cells following a lengthy exposure time (e.g., 30 min to 3 h); it remains unclear if any of these proteins directly contribute to ciliary binding in an effort to overcome mucociliary defense. In this study, we used primary cultured rabbit tracheal epithelial cells (RTEC) as a model for ciliated respiratory epithelium from a natural host of *B. bronchiseptica* and measured the ability of *B. bronchiseptica* strains deficient in individual adhesins and/or toxins to directly bind to cilia on a time scale (<5 min) consistent with overcoming mucociliary defense (14). We show that loss of known individual adhesins (FHA, pertactin, or fimbriae) or toxins (CyaA, DNT) reduced ciliary attachment compared to Bvg⁺ strains but remained significantly higher than Bvg⁻ strains. We also measured the ability of the collectin SP-A to inhibit binding by the phase-locked Bvg⁺ strain of *B. bronchiseptica*. These studies represent the first comparative examination of adherence by single-adhesin or -toxin knockout strains of *B. bronchiseptica* to the ciliary binding of natural host cells and identify a role for a host protein that can disrupt this event.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle medium, fetal bovine serum, Hanks' balanced salt solution (HBSS), and antibiotic-antimycotic (penicillin, streptomycin, and amphotericin B) were obtained from Invitrogen (Carlsbad, CA). Bordet Gengou blood agar plates were obtained from Becton Dickinson and Company (Franklin Lakes, NJ). *B. bronchiseptica* strains were gifts from Peggy A. Cotter (University of California, Santa Barbara), Seema Mattoo (University of California, Los Angeles), and Jeffery F. Miller (University of California, Los Angeles). Human surfactant protein A was a gift from Jeanne M. Snyder and Rebecca E. Oberley (University of Iowa).

Bacterial strains and growth conditions. *B. bronchiseptica* strains used in this study are described in Table 1. Wild-type strain RB50 was isolated from a naturally infected rabbit (4). *B. bronchiseptica* strains RB53, RB54, RB57, RB58, RB63, RBX9, and DF8 were derived from RB50; these derivations are detailed in other studies (1, 4, 5, 16, 29). To create the pertactin-deficient mutant strain SP5, an in-frame deletion in the gene encoding pertactin, *prn*, extending from codon 227 to codon 756, was constructed, leaving the promoter region, transcriptional start site, and signal sequence of *prn* intact (Seema Mattoo, personal

communication). To create the DNT-deficient strain 50 Δ *dnt*, an in-frame, deletion in the gene encoding dermonecrotic toxin, *dnt*, extending from codon 17 to codon 1461 was constructed, leaving the purported promoter and transcriptional site of *dnt* intact (Omsland, A., P. Haynes, L. Bennett, and S. Boitano, submitted for publication). All strains were maintained on Bordet Gengou blood agar plates and, prior to use in binding assays, were grown to log phase under constant shaking at 37°C in Stainer Scholte broth (63 mM L-glutamic acid, 2 mM L-proline, 43 mM NaCl, 3.7 mM KH₂PO₄, 2.7 mM KCl, 1 mM MgCl₂, 0.14 mM CaCl₂ · 2H₂O, 29 mM Tris-HCl, pH 7.6) supplemented with Stainer Scholte supplements (33 mM L-cysteine, 3.6 mM FeSO₄ · 7H₂O, 3.2 mM nicotinic acid, 48.8 mM glutathione, and 227.1 mM ascorbic acid) (46).

Eukaryotic cell culture. RTEC cultures were prepared according to previously described methods (8). Briefly, New Zealand White rabbits were injected in the marginal ear vein with sodium pentobarbital, tracheas were removed, and tracheal epithelial lining was teased away using forceps, sectioned into explants with a scalpel, and seeded onto 15-mm glass coverslips coated with rat-tail collagen. Cultures were incubated at 37°C in a 5% CO₂ environment in Dulbecco's modified Eagle medium supplemented with 0.2% NaHCO₃, 10% fetal bovine serum, and 1% antibiotic-antimycotic. RTEC were from 6- to 11-day-old mice for all experiments. Prior to use, cultures were washed thoroughly with HBSS; 1.3 mM CaCl₂, 5.0 mM KCl, 0.3 mM KH₂PO₄, 0.5 mM MgCl₂, 0.4 mM MgSO₄, 137.9 mM NaCl, 0.3 mM Na₂PO₄, 1% glucose) additionally buffered with 25 mM HEPES, pH 7.4.

***Bordetella* binding assay.** Binding studies were adapted from previously described methods (14). To control for the binding effects of conditioned growth medium that could be different between bacterial strains, *B. bronchiseptica* strains from log phase growth, described above, were collected by centrifugation and resuspended into HBSS to an optical density at 600 nm of between 0.20 and 0.34. RTEC were mounted onto an open chamber on an Olympus IX70 microscope stage and adjusted to monitor actively beating ciliated cells. A 60 \times oil immersion objective with differential interference contrast optics and an additional 1.5 \times magnification zoom lens allowed for the visualization of both bacteria and cilia (14). RTEC cultures were washed thoroughly with HBSS prior to experimentation. At time zero, HBSS was replaced with 1 ml of the appropriate *B. bronchiseptica* strain using a syringe and a 16-gauge needle to limit aggregation prior to host cell interaction. Cultures were subsequently washed at 2 and 3 min from the start of coinocubation with 1 ml HBSS to remove nonadherent bacteria. Throughout the binding assay, individual images were captured via a Photometrics Cascade charge-coupled device camera (Roper Scientific, Tucson, AZ) onto a personal computer using Image Master 3.0 software (Photon Technology, Inc., NJ) at 1 frame/3 s for 5 min. Captured tagged image file images were analyzed frame by frame using QuickTime software. Bacteria were scored as "attached" if they remained in contact with a ciliated cell for at least eight consecutive frames (24 s). Although bacterial aggregation was rare in all strains except RB58, ciliary attachments that included more than one bacterium were scored as a single attachment. Experiments that tested the inhibition of binding by SP-A were similar to those described above; however, both bacterial suspension and RTEC cultures were preincubated for 30 min in HBSS supplemented with 10 μ g/ml native human SP-A or bovine serum albumin (BSA) prior to videomicroscopy.

When SP-A effects were tested on only bacterial suspension or host culture, a 30-s preincubation was used in the untested suspension or culture to maintain SP-A concentration during coincubation experiments. The 30-s preincubation did not affect ciliary binding by itself (data not shown). To ensure the reproducibility of experiments, RTEC tissue cultures derived from individual rabbits were tested for binding with up to five *B. bronchiseptica* strains. All bacterial strains were tested for binding on RTEC tissue cultures derived from at least two and up to four different rabbits.

SEM. RTEC were coincubated with 250 μ l of appropriate *B. bronchiseptica* strain suspended in HBSS for 5 min and then rinsed with 1 ml HBSS to remove nonadhering bacteria. Cultures were fixed in 3% glutaraldehyde in 100 mM cacodylate buffer (CB; pH 7.2). Fixed samples were washed with 144 mM CB, incubated in 150 mM CB containing 1% tannic acid for 1 h, and washed again. Samples were postfixed in 100 mM CB containing 1% osmium tetroxide (pH 7.2), washed, and dehydrated in a series of graded ethanol washes. The samples were then washed with 100% hexamethyldisilazane and allowed to dry overnight. Samples were mounted, sputter coated with 20-nm gold particles, and imaged with an FEI-Phillips XL30 scanning electron microscope at 10 kV. Scanning electron microscopy (SEM) sample fixation and imaging were performed by the Arizona Health Science Center Core Imaging Facility, Arizona Research Labs, Division of Biotechnology. SEM images were pseudocolored to highlight bacteria by using Photoshop (Adobe Systems Software, San Jose, CA).

Statistics. Graphical data are presented as the number of bound bacteria \pm standard error of the mean. Significance between attachments by two different bacterial strains at each time point was determined with Student's *t* test using Prism (GraphPad Software Inc., San Diego, CA). A value of $P < 0.05$ was used for the determination of significant difference between samples.

RESULTS

Ciliary binding by *B. bronchiseptica* Bvg phase-locked mutants. We have previously shown that a wild-type strain of *B. bronchiseptica* (RB50) grown under Bvg⁺ conditions preferentially adhered to the cilia of RTEC cultures within seconds of coincubation, whereas a nonmotile, phase-locked Bvg⁻ mutant derivative of RB50 (RB57) showed limited binding over a 5-min coincubation (14). Since RB50 can modulate between Bvg⁺ and Bvg⁻ phases in response to environmental conditions and because RB57 cells lack the flagella normally associated with the Bvg⁻ phase, we repeated our binding studies with phase-locked Bvg⁺ (RB53) and phase-locked Bvg⁻ (RB54) strains (Fig. 1A). In the ciliary binding assay, RB53 displayed binding characteristics identical to RB50. Within 30 s, RB53 cells were attached to ciliated cells (0.94 ± 0.19 bacteria/host ciliated cell) and binding steadily increased over time (10.12 ± 0.83 at 240 s). RB54 displayed a binding pattern similar to RB57, with less than a single bacterium attached per ciliated cell on average at any time point over the 240-s experiment. Both RB50 and RB53 bound to ciliated cells at significantly higher numbers than either of the Bvg⁻ strains within 30 s of coincubation and throughout the experiment. To further demonstrate ciliary-specific binding and differences between Bvg⁺ and Bvg⁻ binding, SEM images were taken after 5 min of coincubation (Fig. 1B through E). SEM images of RB53 and RB50 both displayed similar affinity for the upper portion of ciliated RTEC, with no bacteria associated with exposed aciliated membrane (Fig. 1B and C). In contrast, the Bvg⁻ strains RB54 and RB57 displayed minimal adherence to either ciliated or aciliated areas of the RTEC culture (Fig. 1D and E). Throughout the following experiments, RB50 and RB54 binding data from Fig. 1 are replotted as references for "full binding" and "minimal binding" efficiency.

Ciliary binding by *B. bronchiseptica* in the presence of SP-A. Cells from the conducting airway epithelium produce a variety of antimicrobial agents to prevent bacterial colonization. One

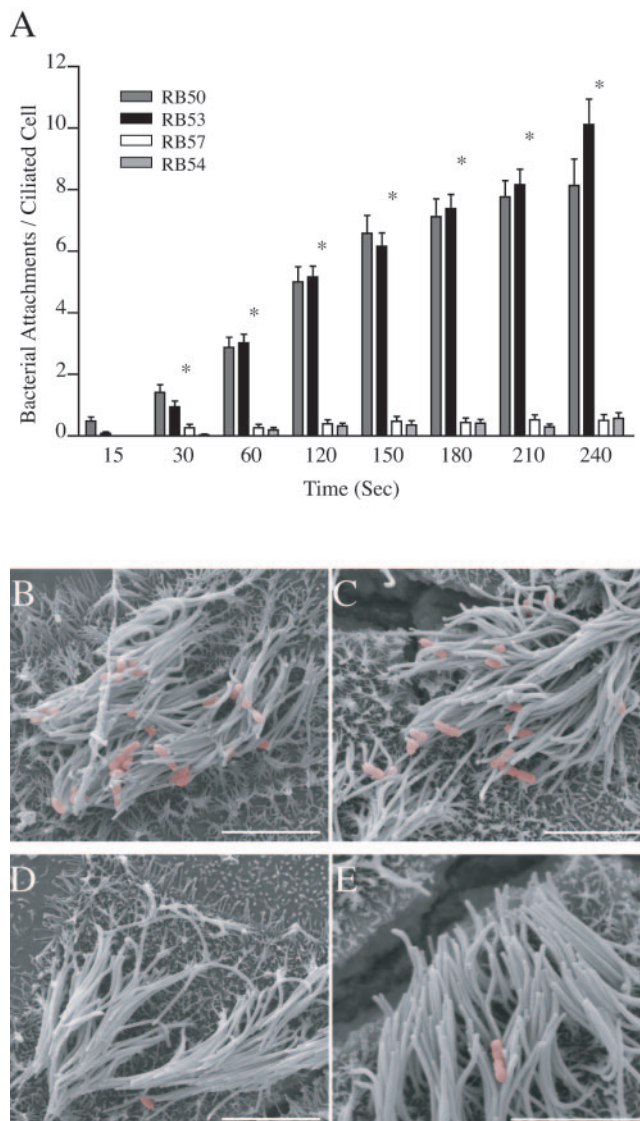


FIG. 1. *B. bronchiseptica* binding to RTEC. (A) Quantification of Bvg⁺ (wild-type RB50 [dark gray bars] and phase-locked RB53 [black bars]) and Bvg⁻ (phase-locked RB57 [white bars] and phase-locked RB54 [light gray bars]) adherence to ciliated RTEC is shown over time. Scanning electron micrographs of RB50 (B), RB53 (C), RB57 (D), and RB54 (E) binding to RTEC after a 5-min coincubation is also shown. Adherence by Bvg⁺ strains RB50 and adherence by RB53 were similar throughout the duration of the experiment and were significantly higher than either Bvg⁻ strain within 30 s of coincubation (*). As seen in the SEM image, bacteria adhered preferentially to the upper portion of the cilia, with Bvg⁺ adhering with greater affinity than Bvg⁻ *B. bronchiseptica*. Adherence is shown \pm standard error with a significance of $P < 0.05$ (*) for $n \geq 23$ for time points at 15 s to 210 s and $n \geq 12$ for the 240-time point. Scale bars represent 5 μ m.

of these agents, SP-A, has been shown to directly interact with pathogenic bacteria, including strains of *B. pertussis*, to aid in bacterial clearance in the airway by opsonization and phagocytosis (30, 44). To test whether SP-A could contribute to innate immunity by reducing ciliary binding by *B. bronchiseptica*, we preincubated suspensions of RB53 and RTEC cultures for 30 min with native human SP-A protein (5 μ g/ml and 10

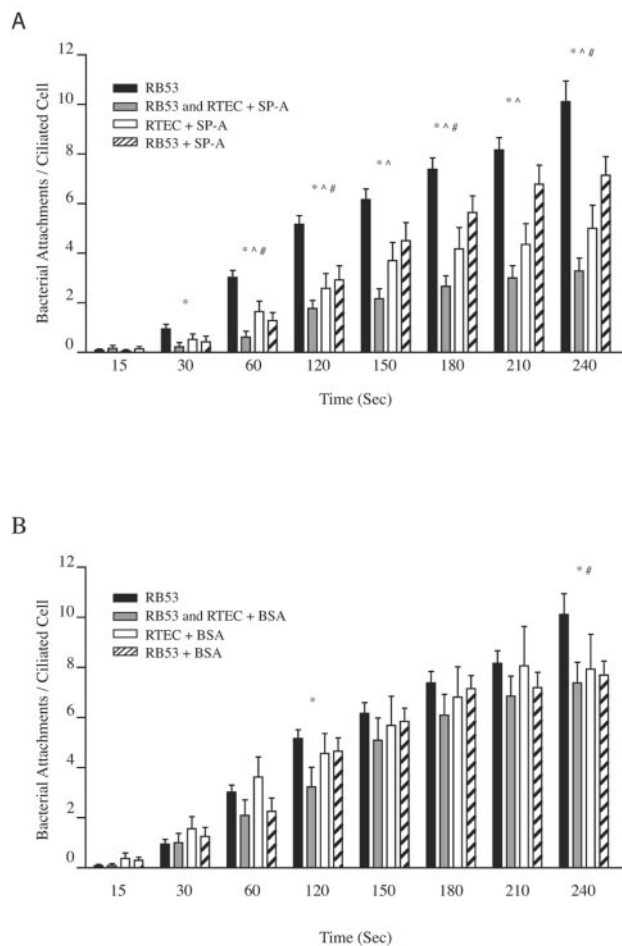


FIG. 2. Ciliary attachment by *Bvg*⁺ *B. bronchiseptica* in the presence of SP-A. The number of phase-locked *Bvg*⁺ (RB53) that bound to ciliated RTEC following preincubation of both RB53 and RTEC cultures (gray bars), RTEC cultures alone (white bars), or RB53 alone (striped bars), all with final concentrations of 10 μg/ml of SP-A (A) or BSA (B), are compared to RB53 binding from Fig. 1 (black bars). Preincubation of both RB53 and RTEC cultures with SP-A significantly reduced RB53 binding within 30 s (*) and remained significantly lower throughout the experiment. Preincubation of RTEC alone with SP-A significantly reduced binding within 60 s (^), continuing throughout the experiment, whereas preincubation of RB53 alone significantly reduced binding at the 60-, 120-, 180-, and 240-s analysis points (#). In contrast, preincubation with BSA to assay a nonspecific protein block resulted in binding patterns most comparable to those for BSA-free binding (Fig. 2B). *, #, and ^ denote statistically significant differences at *P* < 0.05 compared to RB53 data redrawn from Fig. 1, and *n* ≥ 14 for all time points.

μg/ml) and repeated the binding assay. Incubation with 5 μg/ml SP-A did not significantly alter RB53 binding to RTEC cilia (data not shown); however, incubation with 10 μg/ml SP-A significantly reduced RB53 binding within 30 s of incubation, which continued throughout the duration of the experiment (Fig. 2A). In an attempt to determine if SP-A inhibition was bacterial or ciliary specific, the binding assay was repeated after SP-A preincubation with only RTEC cultures or RB53 suspensions. Preincubation of RTEC alone with SP-A significantly reduced the binding capacity of RB53 within 60 s of incubation and throughout the following time points. Pre-

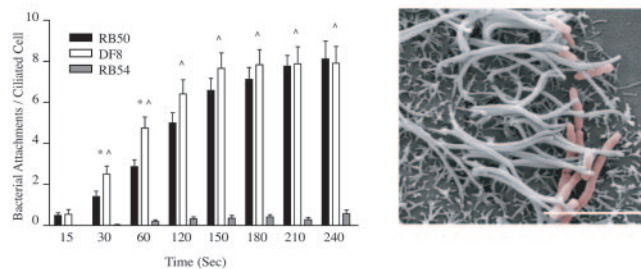


FIG. 3. Ciliary attachment by a *Bvg*⁻, FHA-expressing *B. bronchiseptica* strain. Quantification of DF8 (white bars), a *Bvg*⁻ strain that expresses FHA, compared to RB50 (black bars) and RB54 (gray bars) is paired with a representative SEM graph of binding following 5-min incubation. DF8 bound cilia at a rate similar to that of RB50. Binding was significantly higher than RB50 at 30 and 60 s of incubation. DF8 binding was significantly higher than RB54 from 30 s on. Data for RB50 and RB54 are redrawn from Fig. 1 for comparison. * denotes significantly different binding between DF8 and RB50, and ^ denotes significantly different binding between DF8 and RB54 (*P* < 0.05), with *n* = 24 for DF8 at all time points. Error bars in graphs are means ± standard errors. Scale bar in micrograph represents 5 μm.

incubation of RB53 alone with SP-A reduced overall binding capacity, reaching significance at four of the eight time points tested. As a control for nonspecific protein interference, we repeated the experiments with 10 μg/ml BSA in place of SP-A (Fig. 2B). Preincubation of RTEC alone with BSA did not significantly reduce RB53 binding at any of the time points tested. Although preincubation with BSA of bacterial suspensions alone or a dual preincubation of RB53 and RTEC cultures reduced RB53 adherence, this reduction did not establish significance through consecutive analysis points. Additionally, RB53 adherence following dual preincubation with BSA was significantly higher than adherence following dual preincubation with SP-A at 60 s and again at 150 s where binding differences remained significant throughout the duration of analyses, indicative of a specific block of bacterial adherence by SP-A.

Ciliary binding by *B. bronchiseptica* with ectopic FHA expression. To determine if FHA alone could restore the ability of a *Bvg*⁻ strain to adhere to cilia, we performed the RTEC binding assay with DF8, a *Bvg*⁻ *flaA* mutant strain that ectopically expresses FHA (5). In this strain, the *flaB* and *flaC* promoters were replaced with a comparably strong flagellin promoter, resulting in the expression of the two genes necessary for FHA production in the *Bvg*⁻ phase. In the RTEC binding assay, DF8 adhered to cilia at numbers significantly higher than the *Bvg*⁻ strain RB54 within 30 s and at all other time points tested. Compared to the wild-type strain RB50, DF8 displayed high binding at early time points (30 and 60 s) and bound to ciliated RTEC in similar numbers throughout the duration of the experiment (Fig. 3A). Unlike any of the other strains tested, DF8 displayed a low but significant capacity to bind to aciliated cells and aciliated cell membranes in the RTEC cultures (data not shown). SEM images confirmed high numbers of bacteria associated with cilia and ciliated cells (Fig. 3B). In these images, DF8 exhibited a rod-shaped morphology more typical of the *Bvg*⁻ phase as opposed to the coccus morphology more typically associated with *B. bronchiseptica* in the *Bvg*⁺ phase. From these studies, we conclude that FHA is

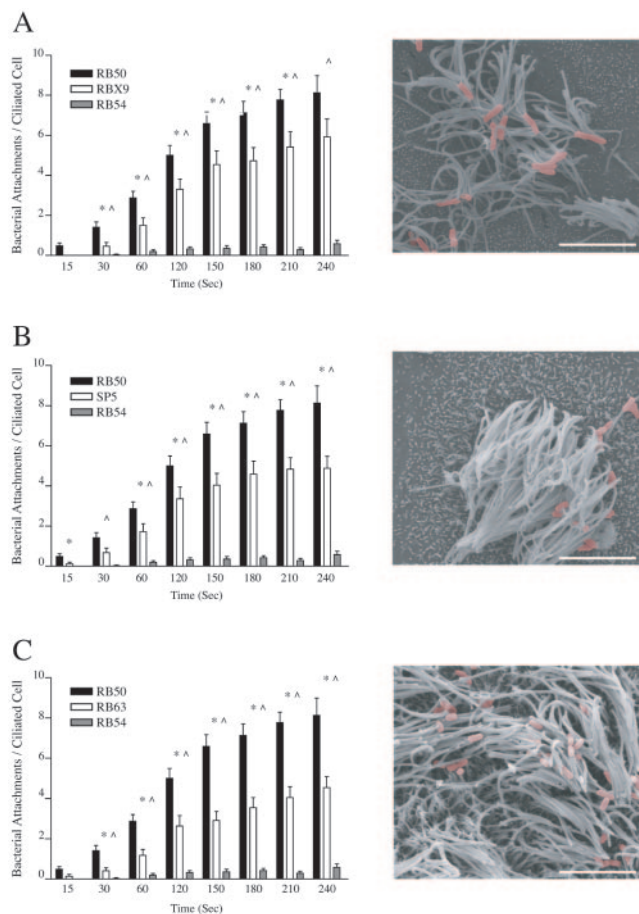


FIG. 4. Ciliary attachment by mutant *B. bronchiseptica* strains missing single-adhesin proteins. Individual graphs quantifying the adherence of single-adhesin knockout strains of *B. bronchiseptica* mutant strains are presented as in Fig. 3. (A) RBX9, a *fha* mutant strain deficient in FHA expression; (B) SP5, a *prn* mutant strain deficient in pertactin expression; (C) RB63, a *fim* mutant strain deficient in fimbrial protein expression. All single-adhesin mutant strains displayed an intermediate binding capacity with a ciliary adherence significantly higher than that of RB54 within 30 s that was maintained throughout the experiments. Binding by RBX9 and RB63 was significantly lower than RB50 beginning at 30 s and continuing throughout the experiment. Binding by SP5 was significantly lower than RB50 at the 15-s time point and again at 60 s, where it remained significantly lower throughout the experiment. Markings are as described in the legend to Fig. 3, except $n \geq 22$ for single-adhesin knockout strains. Scale bar in micrograph represents 5 μm .

sufficient for initial airway epithelial cell attachment in the ciliary binding assay.

Ciliary binding by *B. bronchiseptica* strains lacking single adhesins. To evaluate if any one of the previously identified adhesins—FHA, pertactin, or fimbriae—contributed to the ciliary binding differences observed when wild-type *B. bronchiseptica* cells were grown in Bvg^+ conditions compared to the Bvg^- phase-locked binding, we repeated the RTEC ciliary binding assay with single-adhesin knockout strains (Fig. 4). We first evaluated the role of FHA with the Bvg^+ *fha* mutant *B. bronchiseptica* strain RBX9 (5). RBX9 displayed an intermediate binding pattern, compared to RB50 and RB54, that was significantly different from both within 30 s and that continued

throughout the experiment (Fig. 4A). SEM images verified ciliary attachment of RBX9 to RTEC. In binding assays with SP5, a *prn* mutant strain deficient in pertactin, a similar intermediate binding pattern was observed. SP5 first displayed significantly different binding from RB50 at 15 s and from RB54 at 30 s (Fig. 4B). By 60 s, SP5 binding significance from RB50 and RB54 continued throughout the experiment. SEM images showed ciliary attachment by SP5 to be slightly lower in numbers but with a pattern similar to that observed with RB50. RB63, a *fim* mutant strain deficient in fimbriae (29), also displayed an intermediate adherence compared to RB50 and RB54 (Fig. 4C). RB63 binding was significantly different from RB50 and RB54 within 30 s of coincubation. As above, significance was maintained to completion of the experiment. SEM images taken after a 5-min coincubation of RB63 with RTEC were similar to those seen for the other single-adhesin knockouts described above. Compared among each other, there were no significant differences in attachment to cilia between the three single-adhesin knockout strains at any of the time points tested. Our data show that FHA expression in the Bvg^- phase is sufficient to induce ciliary binding. Additionally, loss of FHA, pertactin, or fimbriae in the Bvg^+ phase reduces ciliary adherence, but loss of any of these single adhesins does not reduce *B. bronchiseptica* binding to the level observed with Bvg^- phase-locked *B. bronchiseptica* during the 5-min analysis developed to test early pathogen-ciliary interactions.

Ciliary binding by *B. bronchiseptica* strains lacking single toxins. *Bordetella* expresses a variety of toxins including CyaA and DNT. Because CyaA has been reported to have adhesive properties (16), we evaluated a *cyaA* mutant *B. bronchiseptica* strain, RB58, in the RTEC binding assay. Similar to the single-adhesin knockout mutants, RB58 displayed significantly reduced ciliary binding compared to RB50 starting at 30 s and significantly increased ciliary binding compared to RB54 starting at 120 s (Fig. 5A). Compared to RBX9 and SP5, RB58 binding to ciliated RTEC was significantly lower within 60 s and throughout the experiment. RB58 binding was also significantly lower than RB63 at 60 s and 120 s; however, this significance was not maintained throughout the experiment. SEM images confirmed a reduced number of RB58 binding sites on ciliated RTEC in culture (Fig. 5B). We also evaluated the *dnt* mutant *B. bronchiseptica* strain, 50 Δdnt , which is unable to express DNT. 50 Δdnt ciliary adherence was significantly higher than RB54 within 30 s of coincubation and throughout the duration of the experiment. Although 50 Δdnt also displayed consistently lower binding than RB50, this difference reached significance at only four of the eight time points tested (Fig. 5C). SEM images showed that 50 Δdnt ciliary binding was comparable to RB50 (Fig. 5D). These data highlight the multifunctionality of the CyaA protein and its contribution to ciliary adhesin, a property not shared by all *Bordetella* toxins, including DNT.

DISCUSSION

In order to successfully infect the conducting airway, primary colonizing bacteria must overcome the inherent innate immunity of the respiratory tract. This includes evading the mucociliary escalator and an environment of antimicrobial factors. We have developed an in vitro model tissue culture sys-

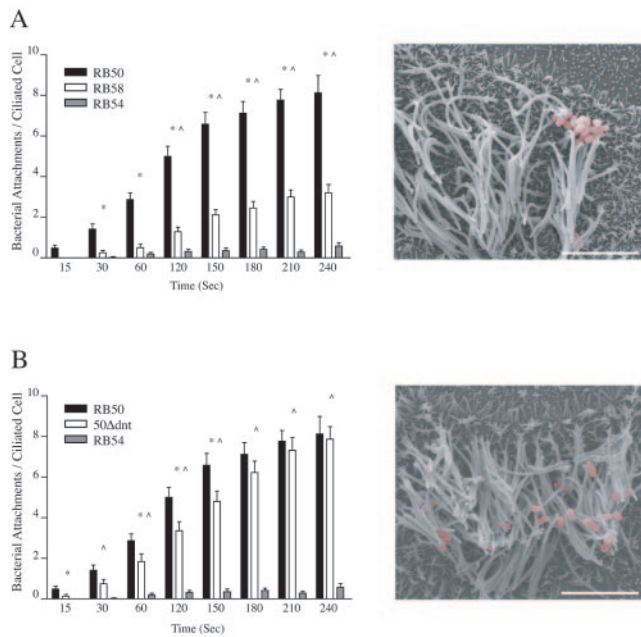


FIG. 5. Ciliary attachment by mutant *B. bronchiseptica* strains missing single-toxin proteins. Individual graphs quantifying the adherence of single-toxin knockout strains of *B. bronchiseptica* are presented as in Fig. 3. (A) RB58, a *cyaA* mutant strain deficient in CyaA toxin; (B) 50Δ*dnt*, a *dnt* mutant strain deficient in DNT expression. RB58 displayed significantly higher binding than RB54 within 120 s and significantly lower ciliary binding than RB50 within 30 s of coincubation. 50Δ*dnt* adherence was also significantly higher than that of RB54 within 30 s; however, significance compared to that for RB50 binding was variable. Markings are as described in the legend to Fig. 3, except $n \geq 24$ for single-toxin knockout strains.

tem to assay one of the mechanisms that *Bordetella* use to overcome the mucociliary escalator—direct binding to cilia on host epithelium (14). We show that an antimicrobial host factor produced in the conducting airway, SP-A, can reduce ciliary binding by *B. bronchiseptica*. We also evaluated roles for several BvgAS-regulated surface virulence factors to mediate adherence to host cell cilia, including but not limited to the adhesins FHA, pertactin, and fimbriae and the toxin CyaA. Using phase-locked Bvg⁺ or Bvg⁻ *B. bronchiseptica*, we confirmed that initial interaction with the ciliated cell via direct binding was dependent on positive BvgAS expression. Additionally, we evaluated the contributions of individual adhesins and toxins in ciliary attachment by comparing ciliary binding by *B. bronchiseptica* strains deficient in single-adhesin or -toxin expression to ciliary binding by wild-type Bvg⁺ or Bvg⁻ phase-locked strains. Our results show that loss of any of three identified adhesins (FHA, fimbriae, or pertactin) or a multifunctional toxin (CyaA) from *B. bronchiseptica* reduces ciliary adherence in comparison to Bvg⁺ strains but does not reduce ciliary adherence to the level observed by Bvg⁻ strains. The ability of *B. bronchiseptica* to express several factors that can directly interact with host cell cilia to overcome mucociliary clearance and the evidence that host cells can block this interaction by the secretion of certain factors suggest that this is an essential determining step in bacterial colonization of the conducting airway.

In addition to coordinated ciliary beat and mucus production, cells of the respiratory epithelium can produce a variety of antimicrobial factors that directly interact with bacteria or other factors and can recruit a full inflammatory response (7). SP-A is an antimicrobial protein that contains both a collagen-like domain and a calcium dependent (C-type) lectin domain (collectins) and is produced by airway epithelial cells in the conducting airway (6, 22, 42). In knockout murine models, loss of SP-A increases susceptibility to bacterial infection (26). This loss of host defense can be through a multitude of antimicrobial mechanisms of action by SP-A, including agglutination, opsonization, modulation of inflammation, or direct inhibition of bacterial growth (6, 9, 30, 52). Although SP-A had limited effects on wild-type *B. pertussis*, strains that expressed mutant lipopolysaccharide were susceptible to enhanced aggregation, permeabilization, and opsonization, leading to monocyte phagocytosis by SP-A protein (44). Although we did not directly test permeabilization or opsonization, SP-A did not cause aggregation of *B. bronchiseptica* (data not shown) but did reduce *Bordetella*-ciliary interactions. The protective effect of SP-A in the ciliary binding assay was best observed when SP-A was preincubated with both bacteria and host cultures; however, a consistent reduction in ciliary binding was observed when either bacterial suspensions or host cultures were preincubated with SP-A. Although we cannot rule out a direct lipopolysaccharide binding activity contributing to the block of ciliary attachment, we speculate that it is more likely that the lectin-like domains inherent to SP-A are blocking exposed sugars important in pathogen/host ciliary attachment. Independent of the mechanism, the role for SP-A in directly preventing the ciliary attachment of *B. bronchiseptica* provides a novel antimicrobial defense in the conducting airway to prevent bacterial colonization.

FHA is a cell surface-associated adhesin that can participate in *Bordetella* binding to a variety of cell types (31, 37–39, 48–50). Although FHA is highly conserved among *Bordetella* spp.—*B. bronchiseptica* FHA and *B. pertussis* FHA are similar in molecular mass and structure and contain shared epitopes—culture supernatants of *B. bronchiseptica* contain less FHA than *B. pertussis* supernatants (21). FHA from *B. pertussis* is the most extensively studied in *Bordetella* and has at least three separate binding activities: a glycosaminoglycan binding site (15, 31), an integrin binding arginine-glycine-aspartate (RGD) sequence (38, 39), and a carbohydrate recognition domain (37, 48). In our studies, the expression of FHA in a Bvg⁻ background is sufficient to restore ciliary binding whereas the loss of expression of FHA in a Bvg⁺ background reduces, but does not eliminate, early ciliary attachment. These data are different than that seen in a previous assay of *B. pertussis* *fha* mutant strain binding to rabbit ciliated cells, where loss of FHA resulted in only 5.8% of the wild-type binding (39). However, our studies examine polarized epithelial cells and focus on ciliary binding in a 5-min experiment, whereas the previous study used a 30-min coincubation with isolated ciliated cells. The use of isolated ciliated cells can also limit ciliary-specific attachment by exposing the basolateral membrane to *Bordetella* and thus may invoke an attachment not normally available to colonizing bacteria. Because mammalian cilia contain a variety of glycosylated proteins (17) and *B. pertussis* FHA binding to human cilia includes a lactose domain (48), the observed FHA-

dependent ciliary binding likely involves the carbohydrate binding domain. We have also shown that ectopic expression of FHA in a Bvg⁻ background (i.e., the DF8 strain) restores ciliary binding and induces aciliated membrane binding by *B. bronchiseptica*. These data are in agreement with the ability of FHA to contribute to aciliated cell binding in a variety of lung epithelial cell lines (50) and may include contributions from all three of the FHA binding domains.

In our ciliary binding assay, loss of fimbriae or pertactin was equivalent to the loss of FHA in the reduction of ciliary binding by Bvg⁺ strains and no single adhesin was necessary for this important early step in *B. bronchiseptica* colonization of the conducting airway. Previous results assaying the role of pertactin in adhesion required extended coincubations and varied depending on the aciliated epithelial cell model used. For example, in studies using a binding assay with a lung laryngeal epithelial cell line (HEp2), *prn* mutant strains had adherence similar to that of wild-type strains of *B. pertussis* (41). In cell models using CHO or HeLa cells, *prn* mutant strains had a decreased binding capacity and host attachment involved an RGD sequence within the pertactin protein (25). Fimbriae are multisubunit extracellular filamentous proteins expressed by most gram-negative pathogenic bacteria that aid in bacterial attachment to host tissues (23). When purified, *B. pertussis* fimbriae or fimbrial subunits can bind to the sulfated sugars chondroitin sulfate, heparin sulfate, and dextran sulfate (11) and mediate *Bordetella* binding to human monocytes, (18) which can help to coordinate FHA binding (19). In cellular assays, *B. pertussis* binding to HEp-2 cells required both fimbriae and FHA; however, fimbriae did not contribute to binding of the bronchial-derived cell line NCI-H₂₉₂ (11, 50). Contrasting results have been found in *B. bronchiseptica* studies using a centrifugation binding assay, where *fim* mutant strains did not show a decrease in aciliated epithelial cell binding, although fimbriae were important to the establishment of colonization in an animal model (29). The reduction of ciliary binding in the *fim* mutant *B. bronchiseptica* strain presented in this study suggests that fimbriae contribute to ciliary attachment and may help to explain the lack of colonization in the animal model. Our data are consistent with a redundant contribution by *Bordetella* adhesins rather than a dominant effect of FHA in ciliary attachment. However, we cannot rule out that the loss of individual adhesins (e.g., pertactin and fimbriae) may indirectly contribute to binding through their interactions with other adhesins (e.g., FHA) and thus a more supportive role in ciliary attachment.

In addition to adhesins, there are several toxins under BvgAS control in *B. bronchiseptica* that could potentially contribute to colonization through adhesion. We tested two of these toxins, CyaA and DNT, in the RTEC binding assay. CyaA is a multifunctional bacterial surface-associated enzyme; the hemolysin domain of CyaA may mediate cellular attachment and allow for internalization of the calmodulin-activated adenylate cyclase toxin domain into host cells, where it catalyzes the uncontrolled conversion of ATP to cyclic AMP (24). In an in vitro assay with human ciliated cells, loss of CyaA from *B. pertussis* significantly reduced ciliary binding (49). Our data show that *cyaA* mutant *B. bronchiseptica* cells were similarly reduced in ciliary binding. Although these data are consistent with a direct contribution of CyaA to bacterial binding of cilia,

previous studies have shown a physical association between CyaA and FHA in *B. pertussis* in vitro (53). Thus, similar to that seen with adhesins, we cannot rule out an indirect effect of CyaA on FHA or other *B. bronchiseptica* adhesins that result in the low binding capacity of this mutant. Purified DNT has been reported to cause dermonecrosis when injected intradermally, enhance DNA and protein synthesis in cell cultures, and alter host cell cytoskeleton via small GTPase activity; however, an in vivo phenotype associated with any of these properties has yet to be established (27). *B. avium* strains have been shown to have reduced attachment to turkey tracheal rings when *dnt*-deficient strains are compared to wild-type strains in a 3-h incubation assay (47). Although there was decreased ciliary binding by 50Δ*dnt* compared to RB50 at some of the time points tested, our results are inconsistent with a DNT contribution to ciliary adhesion and the role for DNT in *Bordetella* pathogenesis remains elusive.

All three adhesin mutant strains and the *cyaA* mutant strain displayed a significantly reduced capacity to bind host cilia in our assay; however, all of these strains displayed significantly high ciliary binding compared to Bvg⁻ strains in the RTEC binding assay. These results suggest that *B. bronchiseptica* relies on multiple adhesins to maximize adherence to the ciliated respiratory epithelium. The redundancy in ciliary adhesiveness highlights the importance of this process in the establishment of colonization in the host. Additionally, we might consider the potential of sequential binding by diverse adhesins that might initiate cell signaling in the host epithelium. Such a paradigm has been shown between *B. pertussis* fimbriae and FHA on human monocytes (18). Further studies are required to determine additional adhesin molecules for *B. bronchiseptica*, their ciliary receptors, and their function in ciliary binding.

In summary, we evaluated the ability of an antimicrobial protein to inhibit the ciliary attachment of *Bordetella* and the relative importance of several *B. bronchiseptica* adhesins and toxins in adherence to cilia. We found that SP-A could decrease ciliary binding by the phase-locked Bvg⁺ strain of *B. bronchiseptica*, representing a novel activity of SP-A in host defense. Additionally, FHA, fimbriae, pertactin, and CyaA all participated in Bvg⁺-dependent ciliary adherence; none of these factors were shown to be necessary for pathogen/host binding. Future studies examining the molecular mechanism of SP-A interference in *Bordetella* binding and identifying additional adhesin molecules on the surfaces of *B. bronchiseptica* cells, or specific interactions between adhesins and toxins that alter ciliary binding, will provide preventative or therapeutic targets against primary colonizing bacteria.

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