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The *Bordetella* Bps Polysaccharide Is Critical for Biofilm Development in the Mouse Respiratory Tract[▽]

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Bordetellae are respiratory pathogens that infect both humans and animals. *Bordetella bronchiseptica* establishes asymptomatic and long-term to life-long infections of animal nasopharynges. While the human pathogen *Bordetella pertussis* is the etiological agent of the acute disease whooping cough in infants and young children, it is now being increasingly isolated from the nasopharynges of vaccinated adolescents and adults who sometimes show milder symptoms, such as prolonged cough illness. Although it has been shown that *Bordetella* can form biofilms in vitro, nothing is known about its biofilm mode of existence in mammalian hosts. Using indirect immunofluorescence and scanning electron microscopy, we examined nasal tissues from mice infected with *B. bronchiseptica*. Our results demonstrate that a wild-type strain formed robust biofilms that were adherent to the nasal epithelium and displayed architectural attributes characteristic of a number of bacterial biofilms formed on inert surfaces. We have previously shown that the *Bordetella* Bps polysaccharide encoded by the *bpsABCD* locus is critical for the stability and maintenance of three-dimensional structures of biofilms. We show here that Bps is essential for the formation of efficient nasal biofilms and is required for the colonization of the nose. Our results document a biofilm lifestyle for *Bordetella* in mammalian respiratory tracts and highlight the essential role of the Bps polysaccharide in this process and in persistence of the nares.

Bacteria belonging to the genus *Bordetella* cause respiratory tract infections in both humans and animals (42). *Bordetella pertussis* is the etiological agent of pertussis, cases of which are steadily increasing in number, even in vaccinated populations (9). It has been proposed that the resurgence of pertussis is due in part to carriage within adolescent and adult populations because of waning immunity (3, 4, 9). *Bordetella bronchiseptica* has a broad host range and naturally infects a wide variety of nonhuman animals. It typically establishes asymptomatic infections but can cause atrophic rhinitis in pigs, kennel cough in dogs, snuffles in rabbits, and bronchopneumonia in guinea pigs (18, 42).

B. bronchiseptica is capable of establishing a chronic and asymptomatic infection and can be harvested from the nasal cavities of rats and mice for extended periods (1, 37). A convincing and frequently proposed hypothesis to explain long-term carriage is the ability of microorganisms to exist as biofilms. Bacterial biofilms are increasingly recognized as important contributors to chronic or persistent diseases. A biofilm is generally defined as a surface-attached population of one or more types of bacteria encased in a polymeric matrix, which can be composed of a number of different macromolecules, including nucleic acids, proteins, and polysaccharides (5). Numerous studies have documented the ability of biofilm bacteria to be recalcitrant to antibiotic treatments and to the host immune system (31, 39, 40, 53).

We and others have recently demonstrated the ability of the three classical *Bordetella* species (*B. pertussis*, *B. bronchiseptica*, and *B. parapertussis*) to form biofilms on abiotic surfaces (27, 45, 50). It has been hypothesized that *Bordetella* biofilm formation may play a role in the pathogenic cycle, specifically in persistence within the nasopharynx (29, 46).

Confocal scanning laser microscopy (CSLM) of nasal tissues harvested from mice infected with these bacteria revealed multilayer clusters of sessile bacterial communities that exhibited distinct architectural features. Scanning electron microscopy (SEM) further revealed the presence of multicellular communities adhered to the ciliated epithelium which appeared to be encased in an opaque matrix-like material.

Although extracellular polysaccharides have been shown to be required for one or more of the steps that lead to in vitro biofilm development (5), clear visualization of a biofilm-associated polysaccharide and direct genetic evidence for the involvement of polysaccharides in the respiratory tract are lacking. We have recently demonstrated the involvement of a polysaccharide locus, *bpsABCD*, in the formation or stabilization of the three-dimensional architecture of mature biofilms formed by *B. bronchiseptica* (46). The Bps polysaccharide is antigenically and biochemically similar to the poly-β-1,6-*N*-acetylglucosamine (called PIA, PNAG, or PGA) group of polysaccharides produce by diverse bacterial species (22, 24, 34, 44, 52, 54). We demonstrate here that in vivo, *Bordetella* biofilms are characterized by extrusion of the Bps polysaccharide. We compared the abilities of a wild-type *B. bronchiseptica* strain and an isogenic mutant derivative (Δ*bps*) to form biofilms in the nose. In contrast to the wild-type strain, the Δ*bps* strain was able to neither form robust biofilms nor persist within the nasal cavity of mice at a later time point. The data thus demonstrate the in vivo biofilm mode of existence for *B. bronchiseptica* and implicate the Bps polysaccharide in efficient biofilm formation in the respiratory tract.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The wild-type *B. bronchiseptica* strain RB50 and the Δ*bps* strain (an isogenic derivative of RB50 containing an in-frame deletion of the *bpsABCD* locus) have been previously described (46, 48). All strains were maintained on Bordet-Gengou (BG) agar supplemented with 7.5% defibrinated sheep blood. *B. bronchiseptica* strains were grown in Stainer-Scholte broth at 37°C.

Animal experiments. Five- to 6-week-old female C57BL/6 mice (Jackson Laboratory) were lightly sedated with isoflurane (Butler) and were intranasally inoculated with either 50 μl of sterile phosphate-buffered saline (PBS) alone or with 5×10^5 CFU of RB50 or the Δ*bps* strain. At designated times postinoculation, mice were euthanized, and the nasal septum was excised, fixed in 10% normal buffered formalin, and processed for microscopy as described below.

For quantification of numbers of bacteria from different tissues, groups of six mice were inoculated with different strains as described above. Five weeks postinoculation, excised tissues were homogenized in PBS and plated onto BG blood agar containing streptomycin (50 μg/ml). Colonies were enumerated after 2 days of growth at 37°C. All animal experiments were carried out in accordance with institutional guidelines and were repeated in duplicate. Statistical analysis was carried using an unpaired two-tailed Student *t* test.

Immunofluorescent labeling of *Bordetella* biofilms formed in vivo. After fixation, tissues were washed with PBS and then blocked with 5% normal donkey serum for 30 min. The tissues were then incubated with polyclonal sera (1:1,000; collected from a rat 30 days after inoculation with a Bvg^+ phase-locked derivative of RB50) (11) for 2 h at room temperature, washed five times with PBS, and subsequently incubated for 2 h at room temperature with a donkey anti-rat secondary antibody conjugated to Alexa Fluor 488 (1:200). Samples were again washed five times and fixed for 30 min in 10% normal buffered formalin to prevent antibody-antigen dissociation during microscopy. Tissues were washed with PBS, permeabilized with 0.1% Triton X-100, and stained for eukaryotic F-actin with a 1:40 dilution of phalloidin conjugated to Alexa Fluor 633 for 30 min. Samples were visualized using a Zeiss LSM510 confocal scanning laser microscope. Images were analyzed to determine biomass and average thickness using the COMSTAT package (23). Statistical analysis was carried out using an unpaired two-tailed Student *t* test.

Detection of Bps polysaccharide. To determine the production of Bps in *Bordetella* biofilms, nasal septa were processed as described above for the visualization of *Bordetella* cells by utilizing the anti-*Bordetella* serum. For detection of Bps, tissues were incubated with affinity-purified goat antibodies specific for deacetylated PNAG (dPNAG) (1:1,000) (35, 41), washed, incubated with secondary donkey anti-goat antibodies conjugated to Texas Red (1:200), and subsequently visualized using a Zeiss LSM510 confocal scanning laser microscope.

SEM of in vivo biofilms. Samples were collected from PBS-inoculated and *Bordetella*-infected mice as described above, washed in PBS, processed for SEM as described previously, and viewed with a Philips SEM-515 scanning electron microscope (45).

RESULTS

***B. bronchiseptica* forms biofilms on murine nasal epithelium.** We hypothesized that the biofilm mode of existence, specifically biofilms in the nasal cavity, promotes persistent *Bordetella* infection. To investigate this, we inoculated mice intranasally with the wild-type *B. bronchiseptica* strain RB50. At designated times postinoculation, the nasal septum was excised and stained with rat anti-*B. bronchiseptica* serum, followed by an anti-rat fluorescent conjugate (Alexa Fluor 488) to detect the bacterial cells, which stained green. In order to better delineate the respiratory epithelium, we stained for eukaryotic actin by utilizing Alexa Fluor 633 conjugated to phalloidin, which stained the epithelium red.

We chose two time points, 15 and 38 days after intranasal inoculation, to demonstrate the presence of biofilms during infection. Within 1 week after inoculation with *B. bronchiseptica*, the entire respiratory tract is colonized. However by 5 weeks, the concentrations of bacteria decline to comparatively low levels throughout the lower respiratory tract but remain high in the nasal cavity of the animals (1, 37). CSLM analysis of nasal septa harvested 15 days postinoculation revealed that *B. bronchiseptica* covered the host epithelium as compact layers of cells that resembled a bacterial mat or lawn with some microcolonies (Fig. 1A, middle panel). In contrast, at 38 days postinoculation the bacterial colonies had expanded vertically and were more heterogeneous, with multiple areas of noncolonized epithelium (Fig. 1A, right panel, and 2B). It is possible that the uncolonized epithelium was either composed of nonciliated cells or represented dispersal of a preexisting biofilm. At this later time point, bacteria were present in large foci, and the biofilms formed were thicker than those formed at 15 days (Table 1).

As a negative control, tissues from PBS-inoculated mice were examined utilizing exactly the same protocol as that used for *B. bronchiseptica*-infected tissues. As shown in Fig. 1A (left panel) and 2A, the nasal septa from these mice displayed little cross-reactivity with the *B. bronchiseptica*-specific antiserum. Sera from PBS-infected mice or the secondary antibody alone did not cross-react with nasal tissues (data not shown). Bacteria have been shown to possess distant homologs of F-actin (15). Thus, it is important to note that in vitro-grown *B. bronchiseptica* did not stain with the antibody conjugated to phalloidin (data not shown).

SEM of nasal tissues. In addition to CSLM, we utilized SEM as an independent means to confirm the presence of biofilms in respiratory tissues. In the case of *B. bronchiseptica*, cell clusters were visible at 15 days after inoculation and were adhered to the ciliated epithelium (Fig. 1B, middle panel). By 38 days postinoculation, bacteria were observed to be in a densely packed multicellular community on the ciliated epithelium (Fig. 1B, right panel). Taken together, the results in Fig. 1 and 2 clearly demonstrate that *B. bronchiseptica* is capable of forming a biofilm in the noses of mammalian hosts.

Production of Bps within the murine nasal cavity. One of the defining characteristics of mature bacterial biofilms is the presence of a matrix which is generally composed of proteins, nucleic acids, and/or polysaccharides (5, 30, 38, 53, 54, 56, 58). We have recently demonstrated the involvement of the Bps polysaccharide in *B. bronchiseptica* biofilm formation in vitro (46). We hypothesized that similar to our previous findings, Bps is produced within *B. bronchiseptica* biofilms and is required for efficient biofilm development in the mouse nasopharynx. Thus, we stained nasal septum specimens harvested from mice infected with *B. bronchiseptica* 38 days postinoculation for bacteria (anti-*Bordetella* serum) and for the Bps polysaccharide (anti-dPNAG). We previously showed that antibodies raised against dPNAG specifically detected Bps in vitro (36, 41, 46). Our results (Fig. 2B) demonstrate that the majority of cells comprising *B. bronchiseptica* biofilms (green) colocalized with the Bps stain (red), thereby suggesting that there was uniform production of the Bps polysaccharide during biofilm maturation in vivo. Most importantly, the production of an extruded polysaccharide along with the presence of distinct architectural features further confirms the biofilm nature of *B. bronchieptica* in the respiratory tract.

As a negative control, we stained tissues from a PBS-infected mouse for the presence of both *Bordetella* and Bps and observed little cross-reactivity (Fig. 2A). Heterologous goat sera did not cross-react with either *B. bronchiseptica* or the host epithelium (data not shown). Additionally, nasal tissues from

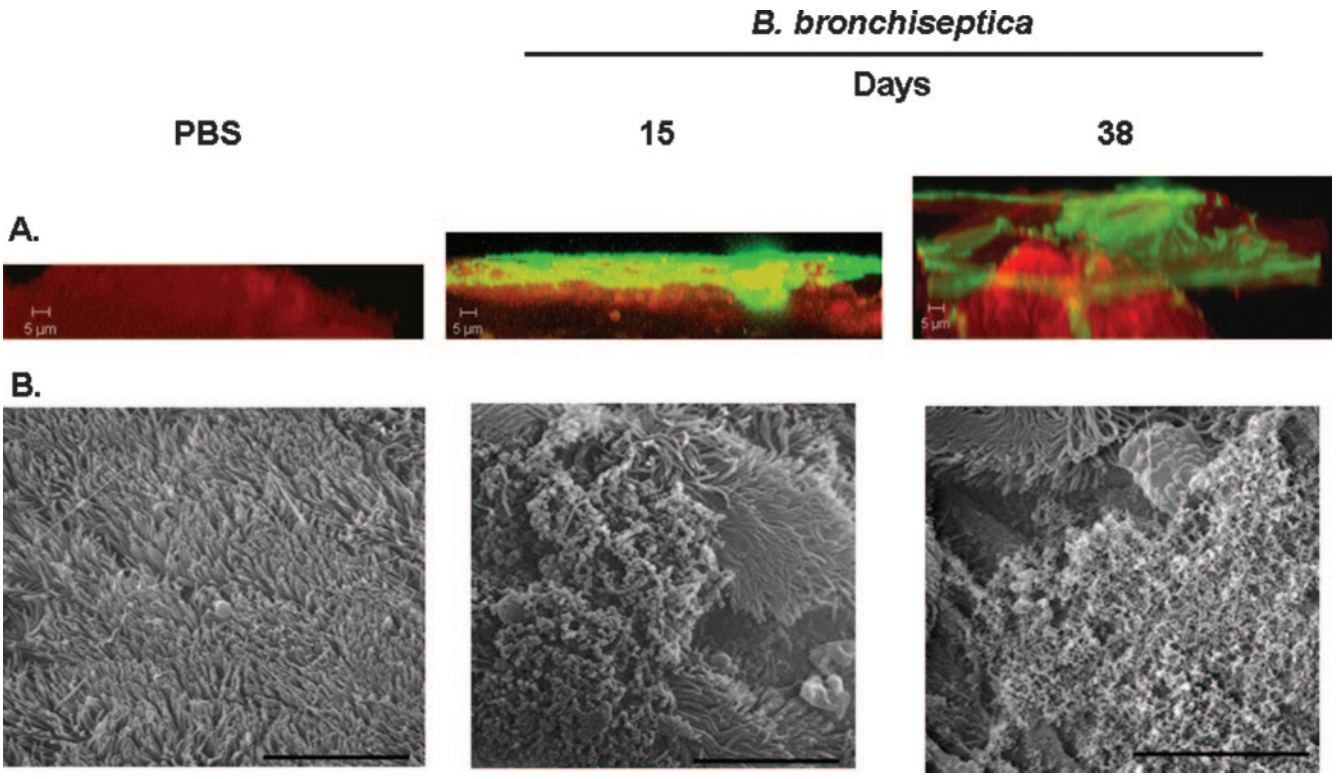

FIG. 1. (A) CSLM of biofilms formed within the murine nasal cavity by *B. bronchiseptica.* C57BL/6 mice were inoculated with either PBS or *B. bronchiseptica* RB50. Nasal septa were harvested at 15 or 38 days postinoculation, immediately fixed, and probed with rat anti-*Bordetella* serum followed by a secondary anti-rat antibody conjugated to Alexa Fluor 488 (which stains bacteria green). To determine the localization of the host epithelium, specimens were stained for F-actin using phalloidin conjugated to Alexa Fluor 633 (which stains the epithelium red) and visualized with CSLM. Each micrograph represents a Z reconstruction. For each specimen, images were obtained from at least five areas of the nasal septum and from at least three independent animals. (B) SEM of *B. bronchiseptica* biofilm formation on nasal septa. Specimens were collected from animals either 15 days or 38 days postinoculation, directly fixed, and processed for SEM as described previously (45). Scale bars = 10 μm.

mice infected with the Δ*bps* strain showed little cross-reactivity with the dPNAG antibody, demonstrating its specificity for detecting Bps in the respiratory tract (Fig. 2C). The low degree of cross-reactivity that remained associated with the Δ*bps* strain was also seen in vitro, where this phenotype can be complemented by restoring the *bpsABCD* locus (46). The complementing plasmid is highly unstable in animals, and we have been unable to obtain any chloramphenicol-resistant bacteria from the animals as early as 2 days postinoculation (data not shown). Thus, it was not possible to use a complemented strain in mice.

TABLE 1. COMSTAT analysis of CSLM micrographs generated from tissues isolated from mice infected with either the wild-type or Δ*bps* strain[a]

Day postinfection	Strain	Biomass[b]		Avg thickness	
		$\mu m^3/\mu m^2$	*P* value[c]	μm	*P* value[c]
15	Wild type	3.270 (4.581)	0.741	5.527 (8.500)	0.447
	Δ*bps*	4.494 (9.057)		2.869 (2.789)	
38	Wild type	3.023 (1.681)	0.0002	12.018 (8.26)	0.0011
	Δ*bps*	0.065 (0.064)		0.094 (0.0953)	

[a] The values are averages for at least seven images from at least two independent animals. The values in parentheses are standard deviations.
[b] Biomass is expressed in $\mu m^3/\mu m^2$ of surface area covered by bacteria.
[c] *P* values were determined using an unpaired two-tailed Student *t* test.

The *bps* locus is essential for *Bordetella* biofilm formation in vivo. Based on our previous studies that clearly documented that the *bps* locus is critical for mature biofilm formation on glass surfaces (46), we hypothesized that it is also required for the production of mature biofilms within the nasal cavity. Thus, we compared the characteristic features of the biofilms formed by the wild-type and Δ*bps* strains by CSLM. We also utilized the COMSTAT software to quantitate the differences in biomass and average thickness between the wild-type and mutant strains (Table 1) (23).

At 15 days, while the wild-type strain was capable of existing as a lawn of cells with some microcolonies, the Δ*bps* strain existed mainly as clusters of cells which covered the majority of the epithelium (compare Fig. 1A, middle panel, to Fig. 3A, left panel). While there appeared to be some differences between the wild-type and Δ*bps* strains microscopically, there were no statistical differences in either the total biomass or the average thickness (Table 1).

In contrast, at 38 days postinoculation, a severe reduction in the ability of the Δ*bps* strain to form biofilms on the nasal septum was observed. The nasal septa harvested from the Δ*bps* strain were to a large extent devoid of bacterial cells (Fig. 2C

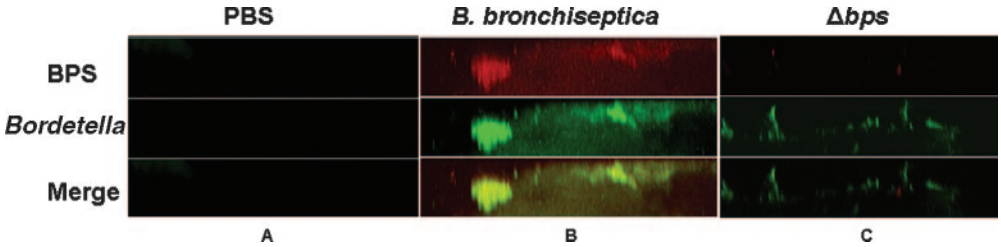

FIG. 2. Bps production in vivo. Nasal septa were harvested from C57BL/6 mice inoculated with (A) PBS, (B) *B. bronchiseptica* (RB50), or (C) an isogenic mutant, Δ*bps*. Samples were collected at 38 days postinoculation and were stained for *Bordetella* (green) as described in the legend to Fig. 1. To detect Bps production (red), specimens were stained using goat anti-dPNAG, followed by anti-goat conjugated to Texas Red. Yellow staining indicates colocalization of *Bordetella* and Bps. Micrographs are Z reconstructions and are representative of at least three independently harvested tissues.

and 3A, right panel). In the regions where the Δ*bps* strain did colonize the epithelium, it was present as very thin spikes and in minute clusters (Fig. 2C and 3A, right panel). Similarly, as quantified by COMSTAT, the Δ*bps* strain formed biofilms whose thickness and biomass were considerably reduced (Table 1).

Since our CSLM and COMSTAT analysis revealed a difference only at 38 days, we analyzed biofilms formed by the Δ*bps* strain at this time point by SEM. The results shown in Fig. 3B demonstrate that the Δ*bps* strain did not form large cell clusters at 38 days and existed only as single cells adhered to the nasal septum.

The *bps* locus promotes persistent colonization of the mouse. Based on the microscopic evaluations described above, we wished to determine the ability of the *B. bronchiseptica* wild-type and Δ*bps* strains to colonize the mouse respiratory tract. At 15 days postinoculation, both the wild-type and Δ*bps* strains colonized the nasal septum, trachea, and lungs to the same degree (Fig. 4). However, at 38 days, considerably lower numbers of bacteria were present in the nasal cavities of animals infected with the Δ*bps* strain ($P \leq 0.005$) (Fig. 4). While no bacteria were recovered from the lungs of one of the six mice inoculated with the wild-type strain at 38 days, the lungs of four animals inoculated with the Δ*bps* strain were cleared of bacteria. For the rest of the animals that did show evidence of lung colonization with the Δ*bps* strain, fewer bacteria were harvested from their lungs than from the lungs of the animals inoculated with the wild-type strain ($P \leq 0.05$) (Fig. 4). Taken together, these results suggest that at least one role for Bps during infection is to allow efficient long-term survival of *B. bronchiseptica* in the respiratory tract.

DISCUSSION

Biofilm development on inert surfaces by a multitude of organisms has been extensively studied, and it is well recognized that bacteria are generally organized as biofilms in nature (13, 47, 55). Although increasing evidence suggests that biofilms may play a principal role in a variety of chronic microbial infections, limited studies have provided direct evidence of the biofilm mode of existence in vivo (2, 21, 25, 32, 33, 51). Parsek and Singh proposed several criteria to define infections caused by biofilms. These criteria were (i) infecting bacteria should be adherent or attached to the substratum, (ii) there should be direct visualization of either bacterial clusters or microcolonies encased in an extracellular matrix, either self-produced or composed of host components, (iii) infections should be localized to a particular anatomical site, and (iv)

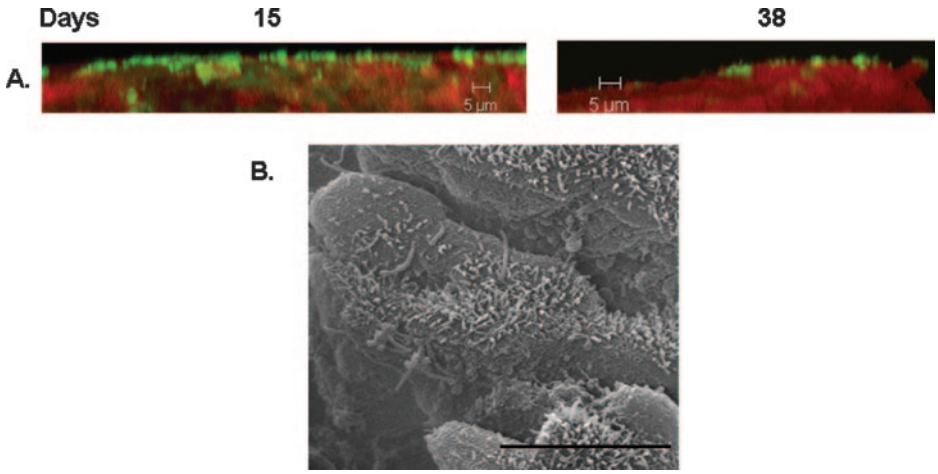

FIG. 3. Involvement of the *bpsABCD* locus in nasal cavity biofilm formation. (A) CSLM analysis of biofilms formed by the Δ*bps* strain. Samples were collected at 15 and 38 days postinoculation and stained as described in the legend to Fig. 1. (B) SEM of Δ*bps*-infected nasal tissue. Samples were collected at 38 days postinoculation and processed as described in the legend to Fig. 1.

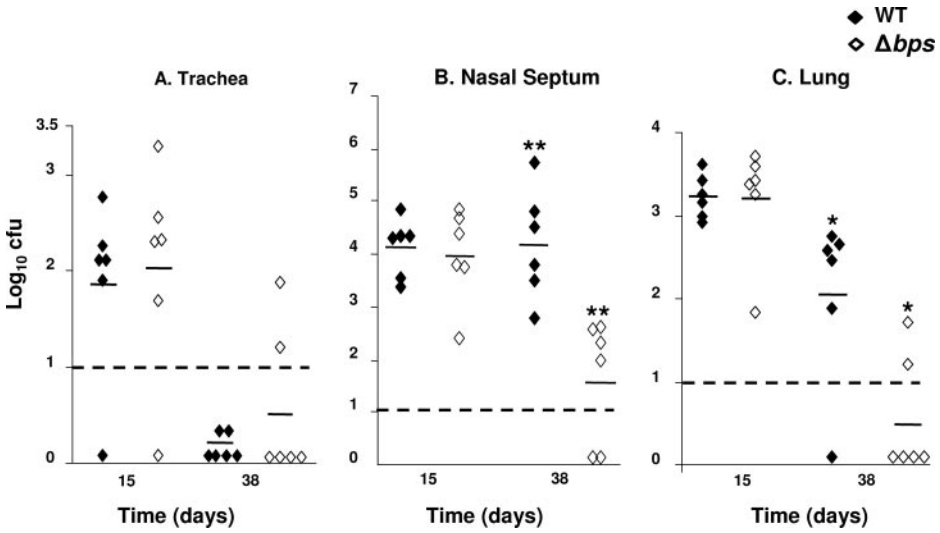

FIG. 4. Colonization of the murine respiratory tract by the wild-type and Δ*bps B. bronchiseptica* strains. Groups of six 6-week-old C57BL/6 mice were intranasally inoculated with 50 μl containing 5×10^5 CFU of either the RB50 (♦) or Δ*bps* (◇) strain. The trachea (A), the nasal septum (B), and two lobes of the lungs (C) were harvested at 15 and 38 days postinoculation and homogenized, and the resident bacteria were enumerated on BG-blood agar plates containing streptomycin. The horizontal bars indicate the numerical mean for each group. The dashed lines indicate the lower limits of detection. The experiment was repeated in duplicate. A statistical analysis was carried out using an unpaired two-tailed Student *t* test. One asterisk indicates that the *P* value is ≤0.05, and two asterisks indicate that the *P* value is ≤0.005.

there should be evidence of recalcitrance of the bacteria to antibiotics compared to their planktonic counterparts (47). In this work, we obtained experimental evidence that there are *B. bronchiseptica* biofilms within the upper respiratory tract. By utilizing CSLM and SEM, we observed adherent clusters (criterion i) of *B. bronchiseptica* that exhibited distinct architectural features (mats, towers, or pillars separated by void spaces) (criterion ii) characteristic of many bacterial biofilms formed in vitro on abiotic surfaces. Notably, the confocal and SEM micrographs of the biofilms observed in vivo were reminiscent of images of in vitro biofilms formed by *Bordetella* spp. on abiotic surfaces, indicating that in vitro studies may serve as good models for examining the roles of different factors in *Bordetella* biofilm development (29, 46, 50). We also obtained evidence that these biofilms are characterized by the production of the biofilm-associated Bps polysaccharide and showed that Bps is essential for efficient biofilm development in vivo (criterion ii).

Although very low numbers of the animal pathogen *B. bronchiseptica* can be isolated at later time points from the lower respiratory tract, this organism mainly establishes asymptomatic and chronic infections of the nose (37). We have been able to isolate *B. bronchiseptica* from the rat nasopharynx 85 days after inoculation (unpublished results), and this bacterium has previously been reported to be present in the nose for the life of an infected animal (criterion iii). Additionally, while *B. pertussis* infection of humans is traditionally associated with the acute disease whooping cough in infants and young children, growing numbers of adolescents and adults are showing milder symptoms and evidence of nasopharyngeal colonization by *B. pertussis* (criterion iii) (10, 20, 49, 57).

Our laboratory has previously shown that compared to their planktonic counterparts, *B. bronchiseptica* biofilms are up to 1,000-fold more resistant to antibiotics, including the clinically relevant antibiotics erythromycin and ciprofloxacin (45). Recently, it has been shown that despite the finding that planktonic *B. bronchiseptica* is highly sensitive to antibiotics, elimination of bacteria from the respiratory tract of infected mice with antibiotics could not be achieved (criterion iv) (28). Thus, results presented here, in combination with previously published reports, strongly document the biofilm nature of *Bordetella* in the respiratory tract.

A clear role for a *Bordetella* virulence factor in the colonization of the mammalian nose has not been reported. The majority of *Bordetella* factors are implicated in the colonization and survival of bacteria in the lower respiratory tract (6, 7, 12, 43). Therefore, it is interesting that despite the presence of numerous virulence factors, deletion of the *bpsABCD* locus can abrogate biofilm development within the nasal cavity. The only strain described to date which does not exhibit nasal cavity colonization is the Bvg^- phase-locked strain which harbors a deletion of the *bvgS* gene and thus does not express any of the Bvg-activated adhesins and toxins (1, 11). We and other workers have previously shown that the BvgAS two-component system is required for biofilm development (27, 45). We are in the process of determining whether expression of Bps is regulated by BvgAS.

Interestingly, while the ability of the Δ*bps* strain to colonize the lungs was impaired, there was no significant defect in colonization of the trachea. In the upper airways, the mucociliary escalator is involved in the clearance of inhaled particles and bacteria from the airways toward the mouth. However, in the lungs, an army of alveolar macrophages and other phagocytic cells phagocytose particles soon after their deposition. Moreover, there are greater numbers of phagocytic cells in the lungs than in the trachea. Thus, one mechanism by which Bps may enhance survival in the lungs is by antagonizing the function of the phagocytic cells.

The ability to detect biofilms formed by *B. bronchiseptica* implies that biofilms have a role during both human and animal infections. This aspect of *Bordetella* physiology and lifestyle has not been examined to date. Several studies have documented the presence of *B. pertussis* in the nasopharynx of both children and adults (3, 4, 8, 17, 19). It is estimated that 20 to 30% of adolescents and adults who have chronic coughs lasting for more than 1 week are colonized with *B. pertussis* in the nasopharynx (9, 14, 16, 26). While *B. pertussis* infections are typically considered acute infections, the course of infection can last for up to 3 months, and a biofilm may allow survival of the bacteria. We hypothesize that the biofilm mode of existence in the respiratory tract, especially in the nasopharynx, allows *B. pertussis* to escape immune defenses, thereby leading to long-term colonization, and may ultimately serve as a reservoir for transmission of the organism to unvaccinated infants and children. We are currently exploring the ability of *B. pertussis* to form biofilms within the murine respiratory tract and the role of the Bps polysaccharide during *B. pertussis* infection.

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