Cross-species protection mediated by a *Bordetella bronchiseptica* strain lacking antigenic homologs present in the acellular pertussis vaccines

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ABSTRACT

The *Bordetella* species are Gram negative bacterial pathogens that are characterized by long-term colonization of the mammalian respiratory tract and as causative agents of respiratory diseases in humans and animals. Despite widespread and efficient vaccination, there has been a world-wide resurgence of pertussis, which remains the leading cause of vaccine-preventable deaths in developed countries. It has been proposed that current acellular vaccines (Pa) composed of only a few bacterial proteins may be less efficacious because of vaccine-induced antigenic shifts and adaptations. To gain insights into the development of newer generation of vaccines, we constructed a *B. bronchiseptica* strain (LPaV) that does not express the antigenic homologs included in any of the Pa vaccines currently in use. This strain also lacks adenylate cyclase toxin, an essential virulence factor and BipA, a surface protein. While LPaV colonized the mouse nose as efficiently as the wild type strain, it was highly deficient in the colonization of the lower respiratory tract and was attenuated in the induction of inflammation and injury to the lungs. Strikingly, and to our surprise, we found that in an intranasal murine challenge model, LPaV elicited cross-species protection against both *B. bronchiseptica* and *B. pertussis*. Our data suggest the presence of immunogenic protective components other than the ones included in the pertussis vaccine. Combined with the availability of the whole genome sequences of many *Bordetella* spp., this study should serve as a platform for the strategic development of the next generation of acellular pertussis vaccines.
INTRODUCTION

Bacteria belonging to the genus *Bordetella* are readily transmitted by either direct contact or through aerosol transmission via respiratory secretions or fomites (6, 30). *Bordetella bronchiseptica* has a broad host range, infecting a wide variety of animals. It is either the etiological agent or is associated with a number of veterinary syndromes such as kennel cough in dogs, atrophic rhinitis and pneumonia in pigs and bronchopneumonia in guinea pigs, rabbits, horses, rats, mice, cats and non-human primates (19). *B. parapertussis* strains can be divided into two genetically distinct types, those which infect humans (*B. parapertussis*<sub>hu</sub>), causing a pertussis-like illness, and those which cause respiratory infections in sheep (*B. parapertussis*<sub>ov</sub>) (15, 16, 42). In contrast, *B. pertussis* is an exclusive human pathogen and causes the acute respiratory disease known as pertussis or whooping cough (6).

Several vaccines against pertussis are currently available. The whole cell vaccines (Pw) consisting of killed whole cell *B. pertussis* organisms were the first generation vaccines to be developed and are still being used in several countries (30). In many industrialized countries, the so-called acellular pertussis vaccines (Pa) that may include up to five antigens (Filamentous hemagglutinin (FHA), Pertactin (Prn), Pertussis Toxin (PT) and two fimbrial proteins (Fim)) have replaced the Pw vaccines (30, 36). These Pa vaccines differ greatly in antigen composition and the amounts of antigens in a vaccine dose (27, 30). Despite the availability of these vaccines, pertussis continues to be a significant cause of morbidity and mortality in infants and young children throughout the world (11). Although vaccination has decreased mortality considerably, *B. pertussis* continues to circulate and persist even in populations that have traditionally...
achieved high vaccination coverage. It is estimated that 20-30% of adolescents and adults who have chronic cough lasting for more than one week are infected with *B. pertussis* (18, 57). Adults and adolescents carrying *B. pertussis* may act as reservoirs for infections in young children in whom the disease can be severe and sometimes lethal (9). In most reported instances of such human-human transmission, infants and children were generally exposed to adults without disease symptoms typical of pertussis but rather only a simple prolonged cough illness (55, 56). Immunity following vaccination or infection is incomplete and wanes in a short time (26).

Several explanations and hypotheses have been suggested for the reemergence of this disease. These include waning immunity following vaccination in the absence of natural and vaccinal boosters, lower efficacy of the current vaccines and changes in the circulating strains because of vaccine-induced adaptation (7, 11). It has been suggested that vaccination may select for vaccine escape mutants that have a different genotype and/or differential antigenic expression pattern than the parent vaccine strains (4). These concerns are heightened by the recent discovery of circulating strains that are deficient in two of the antigens included in the Pa vaccines, PT and Prn (3). Strains containing alterations in the *ptx* promoter, that result in increased production of PT have also been isolated (38). These recent reports thus raise the extreme possibility of the emergence of a strain that could lose all the antigens included in the Pa vaccines, leading to the Pa vaccines being rendered ineffective.

In this report, we constructed a strain of *B. bronchispetica* (LPaV, lacks components of the acellular pertussis vaccine) that harbors deletions in the gene homologs of all of the antigens included in Pa vaccines. The LPaV strain also harbors
deletions in the *cyaA* gene, which encodes for adenylate cyclase toxin (ACT) and *bipA*, encoding for the surface protein *Bordetella* intermediate phase protein A (BipA). While ACT is not included in any of the Pa vaccines, we deleted *cyaA*, because ACT is a critical virulence factor (23, 54). Our reasoning for deletion of the *bipA* gene was based on previous reports of BipA being highly immunogenic in both naturally and experimentally infected animals (17, 53). In a murine model of intranasal infection, LPaV colonized and persisted in the nose of mice. Because this strain did not colonize the trachea and the lungs of mice and induced lower respiratory inflammation compared to the wild type strain, we tested its protective efficacy against two *Bordetella* species, *B. bronchiseptica* and *B. pertussis*. Surprisingly, we found that the mutant strain elicited protection against both species, as defined by reduced colonization in the respiratory tract. These results suggest the presence of immunogenic cross-protective antigens and accentuates the need to develop new generation of pertussis vaccines by identifying novel protective antigens.
MATERIALS AND METHODS

Bacterial strains, media and growth conditions. The wild type B. pertussis strain Bp536 and the B. bronchiseptica strain RB50 used in this study have been described previously (37, 51). The LPaV strain has in frame deletions in five B. bronchiseptica open reading frames, fhaB, prn, cyaA, fim and bipA. The parental strain RB71, an isogenic derivative of RB50 was a gift from Dr. Jeff F Miller. To construct the LPaV strain, we deleted the bipA gene from RB71 as described previously (49). The genotype of this strain was confirmed by PCR.

All the strains were maintained on Bordet-Gengou (BG) agar supplemented with 7.5% defibrinated sheep blood and containing 50 μg/ml of streptomycin. For animal inoculations and other assays, the B. bronchiseptica strains were grown at 37°C in Stainer Scholte (SS) (45) broth on a roller drum. For Bp536, the SS media was supplemented with heptakis (2, 6-di-O-methyl-β-cyclodextrin).

Animal Experiments. 4-6 week old female C57/BL6 mice obtained from Jackson laboratories were lightly anesthetized with isoflurane and intranasally inoculated with 5 x 10^5 CFU of RB50 or the LPaV strain in 25 μl of PBS by deposition onto the nares. The CFU administered were verified by plating an aliquot of the inoculum on BG agar plates containing streptomycin. For time course colonization experiments, groups of 4-5 mice were sacrificed 1, 3, 14 and 30 days post-inoculation and entire nasal septum, 1 cm of the trachea and left lungs were homogenized in PBS and serial dilutions of the suspensions were plated on BG agar containing streptomycin to determine the viable CFU counts. To obtain anti-Bordetella antibodies, sera were collected from infected...
mice 30 days post-inoculation. All experimental procedures were performed in compliance with institutional regulations.

Immunizations. For immunization studies, groups of 4-5 C57BL/6 mice were intranasally immunized at day 0 with $5 \times 10^5$ CFU of LPaV strain in 25 µl of PBS. The control group of mice received 25 µl of sterile PBS. Four weeks post-primary infection with the LPaV strain or inoculation with PBS, mice were challenged with $5 \times 10^5$ CFU of either RB50 or Bp536. For RB50, mice were sacrificed at 7 days and for Bp536, at 3 and 7 days post-challenge. The bacterial burdens in the lungs, trachea and nasal septum were determined as described above.

The colonies of RB50 and the LPaV strains recovered from the mouse respiratory tract were distinguished based on the hemolytic activity and colony morphology. Due to the deletion of the cyaA gene, the LPaV strain is non-hemolytic. To distinguish between Bp536 and LPaV strain bacteria were plated on BG agar plates containing streptomycin and nalidixic acid. LPaV strain is susceptible to nalidixic acid.

For passive immunization, groups of 4-5 mice were intraperitoneally injected with either 200 µl of pooled anti-LPaV convalescent phase serum or sterile PBS. 3-4 h later, mice were intranasally challenged with $5 \times 10^5$ CFU of RB50 in 25 µl of sterile PBS. Mice were sacrificed 7 days post-challenge and colonization levels were determined as described above.

Histopathology. Immediately following sacrifice, the right lungs were harvested and fixed using neutral buffered formalin for 24 h, trimmed and processed for histology as
described previously (50). Hemotoxylin and eosin (H and E) stained sections were examined by light microscopy for evidence of injury in a blind manner by an ACVP board certified veterinary pathologist (Kock). The sections were evaluated for overall cellularity/consolidation, alveolar wall thickness, degree of edema fluid present, and infiltrating neutrophils and macrophages in the airways (alveoli and bronchioles) and interstitium. Each parameter was given a subjective score of 0–5, with 0 being considered normal or unaffected and 5 representing the marked change. Averages from each parameter were added together to give the final inflammatory score for each group.

**ELISA.** Serum antibody responses to RB50 or LPaV were quantified by coating 96-well flat bottom plates with either strain. Overnight cultures of respective strains were diluted to 0.05 OD$_{600}$ /100 µl in 0.1M sodium carbonate/sodium bicarbonate buffer and the plates were incubated at 4°C overnight. Washing and blocking were carried out as described previously (50). Serum from RB50 or LPaV-infected mice was serially diluted and added to the plates as the primary antibody. The total IgG antibodies were detected using HRP-conjugated goat anti-mouse antibody (1:2000). Absorbance at 450nm was determined using Labsystems Multiskan Plus plate reader. The OD$_{450}$ readings were plotted against the corresponding dilutions and end point titers were determined as previously described (50).

**Opsonophagocytosis Assays.** The opsonophagocytosis assays were carried out using the murine macrophage cell line J774A.1 as described previously (50). Briefly,
PBS or different dilutions of sera from naive LPaV-infected mice were heat inactivated at 55°C and incubated with $2 \times 10^6$ CFU of RB50. Subsequently, the bacterial-sera suspensions were added to the macrophage cells at an MOI of 1:10. The plates were incubated at 37°C for 1 h. Following gentamycin treatment, the number of bacteria that were phagocytosed was enumerated by lysing the macrophage cells and plating different dilutions.

Statistical Analysis. Unpaired two-tailed Student $t$ test was used to determine statistical significance between treatment groups. $P$ values $\leq 0.05$ were considered significant. The asterisks indicate the range of $P$ values (one asterisk, $\leq 0.05$; two asterisks, $\leq 0.005$ and three asterisks, $\leq 0.0005$).
RESULTS

A *B. bronchiseptica* strain deleted in five of the known adhesins and toxins colonizes the mouse nose.

*B. bronchiseptica* establishes life-long infection of laboratory animals in the nasopharyngeal cavity (22, 40). In experimental animal models of intranasal infections, *B. bronchiseptica* initially colonizes both the upper and the lower respiratory tract. While it eventually gets cleared from the lower respiratory tract, high numbers of bacteria are recovered from the nasal cavity upto 270 days post-inoculation (22, 40). Previous results have shown that the individual loss of virulence factors, FHA, Fim, Prn, ACT and BipA have little if any effect on the ability of *B. bronchiseptica* to colonize the nasal cavity (12, 28, 31, 39, 49). Since *Bordetella* species express multiple adhesins and toxins, we hypothesized that while the lack of individual factors may not have an effect, the simultaneous absence of these may lead to a defect in nasal colonization. In order to test this hypothesis, we constructed a penta-mutant (LPaV) with in-frame deletions in *prn*, *cyaA*, *fhaB*, *fimBCD* and *bipA*. Immunoblotting of the outer membrane fractions of the LPaV strain using convalescent phase sera from RB50-infected mouse confirmed the absence of these proteins (data not shown). Moreover, *B. bronchiseptica* does not express PT because of inactivating mutations in the *ptx* promoter (21). Thus, LPaV does not produce any of the protein homologs included in the current Pa vaccines.

The colonization kinetics of the mouse respiratory tract by the LPaV strain was compared with that of the wild type strain RB50. Consistent with previous results, RB50 was recovered from the nasal septa, tracheas and the lungs of all the animals at high numbers at 1, 3 and 14 days post-inoculation (Fig. 1) (22). At 30 days post-infection
while RB50 was cleared from the trachea and the lungs, greater than 1000 CFU were recovered from the nasal septum. In contrast, the LPaV mutant displayed a drastic defect in colonization of the trachea and the lungs at all time points examined (Fig. 1). As early as 1 day post-challenge, significantly lower numbers of bacteria were recovered from the lungs and trachea of the LPaV-infected mice than those obtained from these organs of the wild type infected mice (Fig. 1). By 14 days post-infection, the LPaV strain was not recovered from the lungs. Three of five mice completely cleared the LPaV infection from the trachea and in two mice the bacterial load was at the lower limit of detection. However, the ability of this mutant strain to colonize the nasal septum at 14 and 30 days post-infection was indistinguishable from the wild type strain (Fig. 1). These data suggest that concomitant deletion of *fhaB*, *cyaA*, *fim*, *prn* and *bipA* does not significantly affect the ability to persist in the upper respiratory tract.

The LPaV mutant induces less pathology in the infected mouse lung.

In order to determine if decreased colonization of the lower respiratory tract by the mutant strain correlated with reduced pulmonary injury, lungs were collected at 1 and 14 days post-challenge, sections were fixed, processed for H and E staining and examined microscopically. At 1 day post-infection, lungs of RB50-infected mice showed extensive alveolar wall thickening mostly due to infiltration of neutrophils and received a mean pathology score, 4.0 (Fig. 2). At 14 days post-infection, the lungs of this group of infected mice showed enhanced infiltration by macrophages but relatively fewer neutrophils with an average score 3.5. In contrast, lungs from mice infected with LPaV exhibited modest changes with average pathology scores of 1.5 and 1.0 at 1 and 14
days, respectively (Fig. 2). The lungs from PBS-inoculated mice received an average score of 1.0 at both 1 and 14 days post-infection with no evidence of significant injury (Fig. 2). These observations suggest that LPaV elicits reduced injury to the lungs, possibly due to decreased lung colonization.

**Antibody responses to LPaV infection.**

*B. bronchiseptica* elicits a strong antibody response during experimental animal infections (25). Because many of the factors missing from the LPaV strain differentially contribute to the generation of humoral responses against *B. bronchiseptica* (12, 31, 39, 49), we measured the anti-*Bordetella* titers in sera of mice infected with this mutant. ELISAs using RB50 cells as the antigen revealed that LPaV-infected mice had significantly lower anti-*Bordetella* titers than RB50-infected mice at 30 days post-inoculation (Fig. 3). However, the serum antibody responses were similar in both LPaV-infected and RB50-infected mice when LPaV cells were used as the antigen (Fig. 3) thus indicating that despite the absence of five antigens, infection with LPaV induces anti-*Bordetella* antibodies. These data further suggest the presence of other known or yet to be identified antigens in the LPaV strain.

LPaV-specific serum is sufficient to clear *B. bronchiseptica* infection from the lower respiratory tract.

Anti-*Bordetella* antibodies are critical for resolving *Bordetella* infections as well as for vaccine-mediated immune responses (20, 25). Previous studies have demonstrated that passive immunization with sera from RB50-infected convalescent mice leads to the
rapid elimination of *B. bronchiseptica* from the lower respiratory tract (25, 50). Therefore, we investigated if adoptive transfer of sera from LPaV-infected animals will provide protection from colonization with the wild type strain RB50 in the mouse respiratory tract. Pooled sera collected from LPaV–infected mice or sterile PBS was transferred intraperitoneally followed by challenge with RB50. Seven days post-challenge the bacterial burdens in the nose, trachea and the lungs were determined. While mock-treated mice harbored greater than $10^5$ CFU in the lungs, 4 of 5 mice treated with the LPaV serum completely cleared RB50 from the lungs (Fig. 4). Although not as drastic as in the lungs, passive immunization with LPaV sera also resulted in a significant lowering of the bacterial load in the trachea and 2 of 5 mice that received the LPaV serum had no detectable bacteria at this site (Fig. 4). Adoptive transfer of LPaV antibodies did not have a significant effect on nasal colonization by RB50 (Fig. 4). Note that previous studies (25) (50) demonstrate that adoptively transferred anti-*Bordetella* antibodies have no effect on upper respiratory tract colonization by *Bordetella* in mice. These results suggest that in spite of lower titers, antibodies elicited during infection of mice with the LPaV strain are efficient in providing protection in the lower respiratory tract against wild type *B. bronchiseptica* infection.

**Opsonization with anti-LPaV serum enhances phagocytosis of *B. bronchiseptica***. Antibody mediated bacterial uptake by phagocytes is often correlated with protective efficacy of passively transferred immune sera (43, 50). We examined the efficiency of LPaV serum to promote phagocytosis of RB50 by the murine macrophage cell line J774. PBS and sera collected from naïve mice were utilized as negative controls.
was a dose-dependent increase in the uptake of RB50 by J774 cells when opsonized with the LPaV serum as compared to opsonization with either the serum from naïve mice or PBS only (Fig. 5). These results suggest that one of the mechanisms for the observed passive protection mediated by anti-LPaV serum is increased opsonization of B. bronchiseptica for phagocytosis.

Immunization with the LPaV strain elicits cross-protective immunity against both B. bronchiseptica and B. pertussis. Next we investigated the utility of LPaV as a potential live vaccine for B. bronchiseptica. Thirty days post-inoculation with LPaV, mice were challenged with RB50. Bacterial burdens in the lungs, trachea and nasal septum were then determined 7 days post-challenge with RB50. Although LPaV is cleared from the trachea and lungs at 30 days post-inoculation, it is still present in nose at this time-point (Fig. 1). Thus, we utilized hemolysis as a means to distinguish between the colonies of RB50 and LPaV. RB50 produces colonies with a distinct hemolytic zone whereas LPaV results in non-hemolytic colonies because of the deletion of the cyaA gene. Compared to the mock-treated mice, LPaV-vaccinated mice completely cleared RB50 infection from the lungs and trachea and bacteria were not recovered from these organs (Fig. 6A). Similarly, close to a 3 log reduction in the numbers of RB50 colonies was observed from the nose of LPaV-vaccinated mice, compared to that from the nose of mock-treated mice (Fig. 6A). For two of the five vaccinated mice the level of colonization was near the threshold of detection (Fig. 6A). Although there was no significant differences in the growth of RB50 and the LPaV strain in broth cultures (data not shown) and the LPaV strain colonizes
the nose as efficiently as RB50 (Fig. 1), we cannot exclude the possibility that the observed reduction in the numbers of RB50 was because of competition with the resident LPaV bacteria in the nasal cavity at the time of challenge.

Based on the protection observed with RB50, we determined whether LPaV would generate cross protection against the human pathogen *B. pertussis*. Despite the caveat of a *B. bronchiseptica* strain being utilized to examine protection against *B. pertussis*, the *B. bronchiseptica*-mouse model represents a natural-host animal model and is also analogous and representative of human- *B. pertussis* infections (22). To precisely count the number of *B. pertussis* obtained from the nose, bacteria harvested from this site were plated on BG plates containing nalidixic acid and streptomycin. LPaV is susceptible to nalidixic acid. At 3 days post-challenge, the greatest decline in the bacterial load was observed in the trachea, while there was a small but significant decline in the lungs (Fig. 6B). While the colonization of the nose by *B. pertussis* at this time point was highly variable, for two of the LPaV-vaccinated mice, the CFU obtained were at or close to the lower limit of detection. At 7 days post-challenge, all the immunized mice had cleared *B. pertussis* from the trachea. Although at 7 days we did not recover bacteria from the lungs of one of the control mice, when compared to the rest of the mice in this group, the LPaV-immunized mice harbored an average of 50-fold fewer bacteria in the lungs (Fig. 6B). Compared to three days post-inoculation, the burden of *B. pertussis* in the nose of the LPaV-vaccinated mice was further lowered at 7 days post-inoculation and two of the mice had no detectable bacteria at this site (Fig. 6B). These results suggest that intranasal immunization with LPaV provides substantial cross-immunity against both *B. bronchiseptica* and *B. pertussis*. 
Vaccination with LPaV reduces lung pathology in mice challenged with *B. bronchiseptica* and *B. pertussis*.

We also examined the lungs of LPaV-vaccinated mice following challenge with RB50 or Bp536 to determine whether prior inoculation with this strain would reduce pulmonary injury as a result of infection with wild type *B. bronchiseptica* or *B. pertussis*. While the lungs of control mice infected with RB50 had alveolar wall thickening mostly due to the infiltration of mononuclear phagocytes and had an average pathology score of 4.3, the lungs of LPaV-immunized mice demonstrated less injury with a mean score of 1.5 (Fig. 7A). Similarly vaccinated mice that were challenged with Bp536 displayed reduced lung injury with a mean score of 1.6 while the lungs of control mice infected with Bp536 were edematous with an average score of 3.0 (Fig. 7B). These results show that immunization with LPaV strain lowers lung pathology associated with *B. bronchiseptica* and *B. pertussis* infection (Fig. 7).
DISCUSSION

Pertussis is an important and sometimes the only vaccine preventable infectious disease presently increasing in countries with long-standing and very high vaccination coverage. \textit{B. pertussis} continues to circulate by persisting in the human nasopharynx resulting in horizontal transmission (8, 10). Similarly, animals and birds continue to be carriers despite vaccination with the animal pathogens \textit{B. bronchiseptica} and \textit{B. avium}, respectively (19, 44, 52). Animals and birds frequently shed bacteria resulting in outbreaks among herds (14). Previous studies have failed to identify a significant role for the known surface proteins and toxins in nasal colonization (12, 28, 31, 39, 49). We hypothesized that efficient nasopharyngeal colonization by \textit{Bordetellae} involves the coordinated participation of multiple virulence factors. We constructed the mutant strain LPaV with the expectation that concurrent deletion of genes encoding for five of the well-studied surface proteins and toxins would drastically reduce colonization of the upper respiratory tract. However to our surprise, this mutant strain was as effective as the wild type strain in colonizing the mouse nasal cavity (Fig. 1). In contrast, it was essentially cleared from the trachea and the lungs and resulted in reduced pulmonary injury (Figs. 1 & 2).

Encouraged by the observed attenuation in the trachea and the lungs and the resultant minimal damage to the host following infection with LPaV strain, we determined the effectiveness of this mutant as a live vaccine for \textit{B. bronchiseptica}. We found that prior infection of mice with this mutant conferred protection against a wild type strain of \textit{B. bronchiseptica} (Fig. 6A). Immunized animals had a significant albeit weaker humoral immune response compared to animals infected with the wild type
strain (Fig. 3). Adoptive transfer of sera from immunized animals rapidly eliminated *B. bronchiseptica* from the lungs and reduced bacterial burdens from the trachea, suggesting that protection mediated by this strain in the lower respiratory tract is mediated in part by antibodies (Fig. 4). A potential utility of this mutant strain is as a live attenuated vaccine against *B. bronchiseptica* in animals. The currently available vaccines against *B. bronchiseptica* are live, attenuated or heat killed bacteria (2, 28, 32, 46, 48). One of the problems associated with these is that the genetic mutations that result in the attenuation are not known. Under survival pressures in the host including co-infections with other pathogens, there exists the possibility that such vaccine strains might revert to a virulent form (47, 48). Since the LPaV mutant strain carries genetically defined mutations, it may serve as a safer vaccine. However, because of its ability to persist in the nose of the experimentally infected animals the potential for herd-herd transmission of such a vaccine strain exists.

The animal pathogen *B. bronchiseptica* is considered to be the evolutionary progenitor of the human pathogen *B. pertussis* (41). Despite this evolutionary relationship, there are distinct differences in genome sizes and gene expression patterns between these two species. *B. pertussis* has a smaller genome and contains a large number of pseudogenes, many of which have been inactivated by insertion elements, in-frame stop codons, and frameshift mutations (13). Our finding that immunization with LPaV provided cross-species protection against *B. pertussis* (Fig. 6B) is important because this strain does not produce any of the protein-homologs of the components included in the different acellular pertussis vaccines. Not only did we observe decreased colonization of the entire respiratory tract by *B. pertussis* following
immunization with LPaV, there was a significant lowering of the overall lung pathology in immunized mice. It appears that the protection afforded by LPaV against *B. pertussis* is mediated by antigens that are shared between *B. bronchiseptica* and *B. pertussis*. It has been recently demonstrated that a *B. pertussis* strain lacking three of the major toxins PT, dermonecrotic toxin (Dnt) and tracheal cytotoxin (Tct) provided protection against colonization of *B. pertussis* in the lungs (34). *B. bronchiseptica* expresses both Dnt and Tct. Although one cannot directly compare the results from this study and that of ours, it is possible that Dnt and Tct may not play a role in protection mediated by the LPaV strain. We are currently engineering deletions of *dnt* and *tct* in the LPaV strain to examine whether these have any role in protection mediated by this strain. Our laboratory has recently identified a new outer membrane protein *Bordetella* colonization factor A (BcfA) in *B. bronchiseptica* (51) and has shown that immunization with this protein significantly lowered bacterial burden in the respiratory tract (50) making it one of the likely candidate protective antigens in the mutant strain. Our preliminary results suggest the existence of a BcfA homolog in *B. pertussis* and we are currently attempting to identify this protein in *B. pertussis*. Recently, it has been shown that Bps22, a protein secreted by the *Bordetella* type III secretion system, protects mice against *B. bronchiseptica* infection (33). While Bsp22 or other type III components may serve as protective antigens against *B. bronchiseptica* infection, a recent study showed that a *B. bronchiseptica* strain lacking *cyA* and type III secretion provided cross-species protection (28). Since Bp536, the *B. pertussis* strain utilized in this study does not express an active type III secretion complex or the secreted proteins, it makes it unlikely that Bsp22 is mediating the observed protection against *B. pertussis*. Other possible
candidate protective antigens include the iron repressible outer membrane proteins (5), LPS (24) and autotransporters like BrKA (29). Utilizing immunoproteomics, multiple novel antigens from *B. pertussis* extracts that are recognized by immune sera have been recently identified (1). Taken together, results from this study and others, highlight the synergistic contribution of known and yet to be identified proteins in maximizing the protection from *B. pertussis*.

In the past two decades, there has been an increase in the number of pertussis cases reported in many developed countries including the USA (30). It has been discovered that currently circulating isolates of *B. pertussis* carry genotypic changes in some of the vaccine antigens such as Prn and PT (3). Consequently, antigens included in the currently available acellular vaccines may eventually fail to impart efficient protection. Thus, it becomes critical that we remain open to alternatives to Pa vaccine based approaches to pertussis immunization. The use of live attenuated *B. bronchiseptica* and *B. pertussis* strains as vaccines for pertussis has been advocated (35). Similarly, the LPaV strain has the potential to serve as a highly effective and safe complement to current pertussis vaccines by inducing immunity against antigens that are not part of the Pa vaccines. Realistically though, because of safety issues associated with Pw vaccines, it may be difficult for the public health authorities in countries where the Pa vaccines are currently used to return to the use of whole cell vaccines. Observations reported herein thus highlight the need to develop the new generation of pertussis vaccines by the identification and inclusion of additional protective antigens.
ACKNOWLEDGMENTS

We thank Dr. Jeffery F. Miller for the generous gift of the RB71 strain. We thank Haiping Lu for technical assistance. We also thank the two anonymous reviewers for providing a number of discussion points in their critique. Research in the laboratory of R.D. is supported by funds from the NIH (grant no. 1R01AI075081), National Research Initiative Grant no. 2006-35604-16874 from the USDA National Institute of Food and Agriculture, Microbial Functional Genomics Program and the American Heart Association. M.C. is supported by a NIH predoctoral training grant.
Figure Legends

Figure 1. Kinetics of respiratory tract colonization of C57BL/6 mice by RB50 and LPaV strains. Mice were intranasally inoculated with $5 \times 10^5$ CFU of either RB50 (diamonds) or LPaV (squares) strains in 25 $\mu$l droplets. At indicated time points post-inoculation mice were sacrificed and bacterial burden was enumerated in the nasal septum, trachea and lungs. Each symbol represents a single mouse. The dashed line represents the lower limits of detection. Bars represent ± standard deviation. A statistical analysis was carried out using an unpaired two-tailed Student t test to compare the CFU obtained from the respective groups of RB50-infected mice to that of mice inoculated with LPaV strain. The asterisks indicate the range of P values (one asterisk, ≤0.05; two asterisks, ≤0.005 and three asterisks, ≤0.0005).

Figure 2. Infection with LPaV strain induces reduced pathology in mouse lungs. Representative photomicrographs of lungs from mice inoculated with RB50, LPaV or PBS at 1 and 14 days post challenge. H and E,100X.

Figure 3. Serum antibody responses in mice following intranasal inoculation with RB50 or LPaV strain. Total Bordetella specific IgG titters were determined using either RB50 or LPaV whole cells as antigen by ELISA. Values represented are mean titters from 4-7 mice and bars represent ± standard deviation. A statistical analysis was carried out using an unpaired two-tailed Student t test to compare the antibody titters obtained
from sera of RB50-infected mice to that of sera from LPaV-infected mice. The asterisks indicate $P$ value of $\leq 0.0005$.

**Figure 4.** Adoptively transferred anti-LPaV serum confers protection against challenge with RB50 in mice. Mice were intraperitoneally injected with LPaV-specific serum (diamonds) or sterile PBS (squares) three-four hours prior to intranasal challenge with $5 \times 10^5$ CFU of RB50 in a 25 µl volume. Seven days post-challenge, mice were sacrificed and bacterial colonization in the nasal septum, trachea and lungs was determined. Dashed line represents lower limits of CFU detection. Individual symbols represent a single mouse. Black bars represent mean colonization of respective group. Unpaired two-tailed Student $t$ test was used to determine statistical significance. The asterisks indicate the range of $P$ values (one asterisk, $\leq 0.05$; two asterisks, $\leq 0.005$).

**Figure 5.** Opsonization with anti-LPaV serum augments the phagocytosis of RB50 by J774 murine macrophages. Approximately, $2 \times 10^6$ CFU of RB50 was incubated with either 1, 5 or 10 % heat inactivated anti-LPaV serum, 10 % naïve mouse serum or sterile PBS at 37° C for 30 min followed by incubation with $2 \times 10^5$ J774 cells for 1 h. 100 µg/ml of gentamycin was added to kill the extracellular bacteria and subsequently the wells were washed twice with sterile PBS to remove adherent bacteria. The cells were lysed with water and the CFU of phagocytosed bacteria were enumerated as described in the Materials and Methods. Results are representative of two independent experiments performed in four replicates. Bars represent ± standard deviation.
Statistical analysis was carried out using an unpaired two-tailed Student $t$ test. Asterisks indicate the $P$ value of $\leq 0.005$.

**Figure 6. Intranasal immunization with LPaV strain protects mice against challenge with *B. bronchiseptica* and *B. pertussis*.**

(A) Groups of 5 mice were intranasally inoculated with $5 \times 10^5$ CFU of LPaV strain (diamonds) or sterile PBS (squares). On day 30 post-immunization mice were challenged with $5 \times 10^5$ CFU of RB50 and bacterial numbers were determined 7 days post-infection.

(B) Mice were intranasally inoculated with $5 \times 10^5$ CFU of LPaV strain (diamonds) or sterile PBS (squares). 30 days post-immunization mice were challenged with $5 \times 10^5$ CFU of Bp536 and bacterial burdens in the respiratory tract were enumerated 3 and 7 days post-infection. Each symbol represents a single mouse. The dashed line represents the lower limits of CFU detection. Black bars represent mean colonization of respective groups. Statistical significance was determined by unpaired two-tailed Student $t$ test. The asterisks indicate the range of the different $P$ values (one asterisk, $\leq 0.05$; two asterisks, $\leq 0.005$ and three asterisks, $\leq 0.0005$).

**Figure 7. Immunization with the LPaV strain reduces lung pathology in *B. bronchiseptica* - and *B. pertussis*- infected mice.**

Mice were immunized with LPaV strain or sterile PBS and challenged with RB50 (A) or *B. pertussis* (B) as described above. On day 7 post-challenge, lungs were harvested fixed and processed for H & E staining. The sections were examined and scored for
pathology in a blinded manner. Representative H & E stained lung sections are shown with the mean pathology score for each group.

(A) The LPaV-RB50 image has prominent bronchiole-associated lymphoid tissue, but the airways are generally clear and the alveolar walls are fairly normal (pathology score 1.5). In contrast, the lung from the PBS-RB50 mouse has diffuse alveolar wall thickening and airway inflammation (pathology score 4.3). H and E, 100X.

(B) The LPaV-Bp536 image shows prominent bronchiole-associated lymphoid tissue, patchy alveolar edema and minimal inflammation (pathology score 1.6), while that of the PBS-Bp536 mouse is markedly infiltrated by inflammatory cells and edema fluid, with reduction of clear airway space (pathology score 3.0). H and E 100X.
REFERENCES


Fig. 1

![Graphs showing bacterial counts in different tissues](image)

- **Nasal septum**
  - Log10 CFU vs. Days post challenge
  - RB50 and LPaV

- **Trachea**
  - Log10 CFU vs. Days post challenge
  - RB50 and LPaV

- **Lungs**
  - Log10 CFU vs. Days post challenge
  - RB50 and LPaV
Fig. 2

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Fig. 3
Fig. 5
Fig. 6A

![Graph showing Log$_{10}$ CFU of RB50 for Nasal Septum, Trachea, and Lungs with different conditions (LPaV-RB50, PBS-RB50).](image-url)
Fig. 6B

3 days

Log CFU of Bp536

7 days

Nasal Septum Trachea Lungs

LPaV-Bp536

PBS-Bp536
Fig. 7A

LPaV- RB50

Average Total Score 1.5

PBS- RB50

Average Total Score 4.3
Fig. 7B

LPaV- Bp536  
Average Total Score 1.6

PBS- Bp536  
Average Total Score 3.0