

Contribution of *Bordetella bronchiseptica* Filamentous Hemagglutinin and Pertactin to Respiratory Disease in Swine^{∇†}

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***Bordetella bronchiseptica* is pervasive in swine populations and plays multiple roles in respiratory disease. Most studies addressing virulence factors of *B. bronchiseptica* are based on isolates derived from hosts other than pigs. Two well-studied virulence factors implicated in the adhesion process are filamentous hemagglutinin (FHA) and pertactin (PRN). We hypothesized that both FHA and PRN would serve critical roles in the adhesion process and be necessary for colonization of the swine respiratory tract. To investigate the role of FHA and PRN in *Bordetella* pathogenesis in swine, we constructed mutants containing an in-frame deletion of the FHA or the PRN structural gene in a virulent *B. bronchiseptica* swine isolate. Both mutants were compared to the wild-type swine isolate for their ability to colonize and cause disease in swine. Colonization of the FHA mutant was lower than that of the wild type at all respiratory tract sites and time points examined and caused limited to no disease. In contrast, the PRN mutant caused similar disease severity relative to the wild type; however, colonization of the PRN mutant was reduced relative to the wild type during early and late infection and induced higher anti-*Bordetella* antibody titers. Together, our results indicate that despite inducing different pathologies and antibody responses, both FHA and PRN are necessary for optimal colonization of the swine respiratory tract.**

Respiratory disease in pigs is the most important health concern for swine producers today. According to the 2006 NAHMS survey, respiratory disease was the greatest cause of mortality in swine, accounting for 53.7% of nursery deaths and 60.1% of deaths in grower/finisher pigs (68). *Bordetella bronchiseptica* is widely prevalent in swine populations and contributes to multiple pathologies in respiratory disease. In very young pigs it causes severe bronchopneumonia with high morbidity and, if untreated, mortality. It is a primary etiologic agent of atrophic rhinitis, causing a moderate to mild reversible form, and promotes colonization by toxigenic strains of *P. multocida*, usually leading to severe, progressive atrophic rhinitis (11, 12). *B. bronchiseptica* is frequently found in nasal turbinates and lung lesions of fattening pigs who may not exhibit clinical signs of respiratory disease. Nonetheless, field surveys document that subclinical pneumonia can result in substantial economic losses due to slower weight gain, increased days to market, and reduced feed efficiency (4, 22). In addition, *B. bronchiseptica* infections increase the severity of respiratory disease associated with other bacterial and viral pathogens and is thus a main contributing agent in porcine respiratory disease complex, a multifactorial disease state that is consistently listed as a top research priority by the National Pork Board (3, 6, 7, 10, 11, 72).

Infection begins with colonization of the ciliated epithelial

cells of the upper respiratory tract. Two well-studied *Bordetella* virulence factors implicated in the adhesion process are filamentous hemagglutinin (FHA) and pertactin (PRN). Both FHA and PRN are regulated by the BvgAS signal transduction system, which controls the expression of virulence determinants involved in the *Bordetella* infectious cycle (14). Numerous in vitro studies have demonstrated that FHA functions as an adhesin and contains several different binding domains (2, 16, 25, 27, 28, 40–42, 58, 63, 66–67, 69–71). These domains include a heparin-binding domain that facilitates binding to sulfated polysaccharides (23), a carbohydrate-recognition domain that promotes binding to ciliated epithelial cells of the respiratory tract and macrophages (52), and an Arg-Gly-Asp (RGD) domain. The RGD domain has been shown to play a key role in the upregulation of intercellular adhesion molecule 1 by epithelial cells, through an NF- κ B signaling pathway, by interacting with very late antigen-5 (28, 29). This RGD domain also plays an important role in the upregulation of CR3 binding activity by interacting with the leukocyte response integrin/integrin-associated protein located on monocytes and macrophages (27). In addition, FHA of *B. bronchiseptica* has been shown to be required for colonization of the rat trachea (16).

PRN belongs to the type V autotransporter protein family and, similar to FHA, contains an RGD domain as well (18). Several in vitro studies have demonstrated PRN to function as an adhesin (19, 32, 36, 71); however, an exact host receptor has not been identified. The role of PRN as a protective immunogen is more clearly defined. Active immunization with purified PRN has been shown to provide protection against mortality and reduce pathology and lung colonization in mice and pigs challenged with *Bordetella*, and passive transfer of a PRN-specific monoclonal antibody has also been shown to provide protection in mice (33, 43, 46, 60).

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The overwhelming majority of studies addressing virulence factors of *B. bronchiseptica* are based on isolates derived from hosts other than pigs. Swine isolates of *B. bronchiseptica* possess unique genetic and phenotypic traits relative to isolates from other host species (20, 44, 55). Recent experiments in rats demonstrate that attachment is a multifactorial process (16, 40), and this is also likely to be true in swine. However, no definitive data exist with respect to swine, either in vivo or with swine tissue or cells in vitro. In this report, we investigate the role of FHA and PRN in *Bordetella* pathogenesis in swine, by constructing two mutants containing an in-frame deletion of the FHA or the PRN structural gene in KM22, a virulent *B. bronchiseptica* swine isolate. We compare both of these mutants to KM22 for their ability to mediate adherence in vitro and to colonize and cause disease during respiratory infection in swine.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. bronchiseptica* strain KM22, a virulent swine isolate, and strains TN27 and TN28, *fhaB* and *prn* deletion mutants of KM22, respectively, were maintained on Bordet-Gengou (BG) agar (Difco, Sparks, MD) supplemented with 10% sheep blood. *B. bronchiseptica* strains RB50 and RBX9 have been previously described (16). Liquid culture bacteria were grown at 37°C to mid-log phase in Stainer-Scholte broth. *Escherichia coli* OneShot TOP10 (Invitrogen, Carlsbad, CA) was used for all cloning steps, and *E. coli* SM10 λ pir was used to mobilize plasmids into *B. bronchiseptica* KM22. When appropriate, antibiotics were included at the following concentrations: carbenicillin, 100 μ g/ml; chloramphenicol, 30 μ g/ml; and streptomycin, 40 μ g/ml.

Cloning and construction of *B. bronchiseptica* KM22 mutants. Strain TN27, containing an in-frame deletion of the *fhaB* gene, was constructed as follows. A 1,625-bp DNA fragment extending from codon 3637 of *fhaB* and encoding the downstream region of *fhaB* was amplified from KM22 genomic DNA by PCR using the primers 5'-GCTAGCCACTTCGAGAACGTTTCAGCCA-3' and 5'-A GTACTGAGGATATCGGCGGCTAGGATCC-3', which were designed such that NheI and SacI sites would be generated at the 5' and 3' ends, respectively. The resulting PCR product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA) to create pTN1. The insert of pTN1, containing the downstream region of *fhaB*, was cloned into pUC19 (New England Biolabs, Ipswich, MA) via EcoRI and SacI sites to obtain pTN2. A 1,280-bp DNA fragment encoding the upstream region of *fhaB* and extending to codon 13 was amplified from KM22 genomic DNA by PCR using the primers 5'-AGTACTGGCGTGCCTACTAACGGAAG-3' and 5'-GCCAAACGCAACAATCTGCCTAGGATCC-3', which were designed such that SacI and BamHI sites would be generated at the 5' and 3' ends, respectively. The resulting PCR product was cloned into pCR2.1, and the insert, containing the upstream region of *fhaB*, was excised by SacI-BamHI digestion and cloned into SacI-BamHI digested pTN2 to create pTN4. The DNA fragment containing both the upstream and the downstream regions of *fhaB* from pTN4 was cloned into pDONR201 (Invitrogen) to obtain pSMS9 by an adapter-PCR and site-specific recombination using the Gateway cloning system (Invitrogen). The chloramphenicol resistance cassette from pKRP-10E (49) was then cloned into pSMS9 via SacI site to obtain pSMS12. pSMS9 and pSMS12 were mixed with pABB-CRS2 (64), which confers sucrose sensitivity and carbenicillin resistance, and digested with NcoI and NotI to obtain pSMS13 and pSMS14, respectively, using the Gateway cloning system. pSMS14 was introduced into *E. coli* SM10 λ pir (65) and transconjugated into KM22, as previously described (17). Briefly, transconjugates were selected on medium containing streptomycin and chloramphenicol. Cells from a second recombination event, resulting in the excision of the plasmid, were then selected on medium containing sucrose. The resulting mutant strain, harboring the chloramphenicol resistance cassette in place of *fhaB*, was designated TN25. pSMS13 was introduced into *E. coli* SM10 λ pir (65) and transconjugated into TN25, as previously described (17). Here, transconjugates were selected on medium containing streptomycin and carbenicillin, and then cells from a second recombination event, resulting in the excision of the plasmid, were selected on medium containing sucrose. The resulting mutant strain containing an in-frame deletion in *fhaB*, was designated TN27. To confirm the in-frame deletion of the *fhaB* gene, DNA sequence analysis was performed on a 4,021-bp DNA fragment, extending beyond both the

upstream and the downstream regions of *fhaB*, amplified from TN27 genomic DNA by PCR using the primers 5'-GCGCCCGCTGGATTTGAGG-3' and 5'-TGGGGTAGCGCAGCACTTTTG-3'.

Strain TN28, containing an in-frame deletion of the *prn* gene, was constructed as follows. A 1,425-bp DNA fragment encoding the upstream region of *prn* and extending to codon 11 was amplified from KM22 genomic DNA by PCR using primers 5'-AAGCTTCCAACCGGCTTAAATCCTTC-3' and 5'-AGTACTC GCCTTGACAATGCGTGACAGAG-3', which were designed such that HindIII and SacI sites would be generated at the 5' and 3' ends, respectively. The resulting PCR product was cloned into pCR2.1 to create pTN8. To remove an internal SacI site at position 251, pTN8 was digested with HindIII and self-ligated to obtain pTN9. A 1,217-bp DNA fragment extending from codon 742 of *prn* and encoding the downstream region of *prn* was amplified from KM22 genomic DNA by PCR using the primers 5'-AGTACTCTGCGCGCCAGCCG CCTCGAAAATGACT-3' and 5'-GCTAGCCAAGCTGCCGCGGAAGACC AC-3', which were designed such that SacI and NheI sites would be generated at the 5' and 3' ends, respectively. The resulting PCR product was cloned into pTN9 via SacI and NheI sites to create pSMS6. The chloramphenicol resistance cassette from pKRP-10E (49) was then cloned into pSMS6 via SacI site to obtain pSMS15. The DNA fragment containing both the upstream and downstream regions of *prn* from pSMS6 was cloned into pDONR201 (Invitrogen) to obtain pSMS27 by an adapter-PCR and site-specific recombination using the Gateway cloning system. The DNA fragment containing the upstream region of *prn*, the chloramphenicol resistance cassette, and the downstream region of *prn* from pSMS15 was cloned into pDONR201 to obtain pSMS28 by an adapter-PCR and site-specific recombination using the Gateway cloning system. pSMS27 and pSMS28 were mixed with pABB-CRS2 (64), which confers sucrose sensitivity, and digested with NcoI and NotI to obtain pSMS37 and pSMS38, respectively, using the Gateway cloning system. pSMS38 was introduced into *E. coli* SM10 λ pir (65) and transconjugated into KM22, as previously described (17). Transconjugates were selected on medium containing streptomycin and chloramphenicol. Cells from a second recombination event, resulting in the excision of the plasmid, were then selected on medium containing sucrose. The resulting mutant strain, harboring the chloramphenicol resistance cassette in place of *prn*, was designated TN26. pSMS37 was introduced into *E. coli* SM10 λ pir (65) and transconjugated into TN26, as previously described (17). Transconjugates were selected on medium containing streptomycin and carbenicillin and then cells from a second recombination event, resulting in the excision of the plasmid, were selected on medium containing sucrose. The resulting mutant strain, containing an in-frame deletion in *prn*, was designated TN28. To confirm the in-frame deletion of the *prn* gene, DNA sequence analysis was performed on a 4,769-bp DNA fragment, extending beyond both the upstream and downstream regions of *prn*, amplified from TN28 genomic DNA by PCR using the primers 5'-TGCCGGAATTCAT CTCGTC-3' and 5'-ATCTTCTCGCGCCATTATCA-3'.

Western blots. *B. bronchiseptica* strains were grown to logarithmic phase in Stainer-Scholte broth supplemented with 40 μ g of streptomycin/ml. Lysates containing 10⁸ CFU of the indicated strain were run on 4 to 12% Novex Nu-PAGE Bis-Tris Gel (Invitrogen) with morpholinepropanesulfonic acid buffer. Protein was then transferred to a nitrocellulose membrane and divided into three sections: (i) the top of the blot to between 80- and 110-kDa bands of marker, (ii) between the 80- and 110-kDa bands to just above the 30-kDa band, and (iii) just above 30-kDa band to the bottom of the blot. Each section was probed for either FHA, PRN, or BvgA using the previously described antibodies X3C and BPE3 (5, 35) and 63/8, a BvgA-specific monoclonal antibody kindly provided by Scott Stibitz. Membranes were then visualized with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ) using the AlphaInnotech FluorChem IS-9900 charge-coupled device imaging system.

Quantitative real-time PCR. Mid-log-phase cultures of KM22, TN27, and TN28 were diluted in Stainer-Scholte broth supplemented with 40 μ g of streptomycin/ml to obtain a starting optical density at 600 nm (OD₆₀₀) of 0.02, in triplicate, and grown at 37°C with shaking at 275 rpm until an OD₆₀₀ of 0.8 was reached. Bacteria were harvested, and total RNA was extracted with TRIzol (Invitrogen), treated with RNase-free DNase I (Invitrogen), and purified by using RNeasy columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNase-treated total RNA (1 μ g) from each biological replicate was reverse transcribed using 300 ng of random oligonucleotide hexamers and SuperScript III RTase (Invitrogen) according to the manufacturer's protocol. The resulting cDNA was diluted 1:1,000, and 1 μ l of this dilution was used in quantitative PCR reactions containing 300 nM primers and 2 \times SYBR green PCR master mix (Applied Biosystems, Foster City, CA) using an Applied Biosystems 7300 real-time PCR detection system. All primers were designed by using Primer Express software (Applied Biosystems) and are listed in Table S1 in the supplemental material. To confirm the lack of DNA contamination,

reactions without reverse transcriptase were performed. Dissociation curve analysis was performed for verification of product homogeneity. Threshold fluorescence was established within the geometric phase of exponential amplification and the threshold cycle (C_T) value for each reaction was determined. The C_T value from all three biological replicates for each strain was compiled, and the 16S RNA amplicon was used as an internal control for data normalization. The fold change in the transcript level was determined by using the relative quantitative method ($\Delta\Delta C_T$) (38).

Microarray analysis. Fluorescently labeled cDNA from KM22, TN27, and TN28 were prepared using 5 μ g of DNase-treated total RNA from each biological replicate as previously described (45). The two differentially labeled reactions to be compared, TN27 versus KM22 and TN28 versus KM22, were combined and hybridized to a *B. bronchiseptica* long-oligonucleotide microarray as previously described (45). Slides were then scanned by using a GenePix 4000B microarray scanner, and analyzed with GenePix Pro software (Axon Instruments, Union City, CA). Spots were assessed visually to identify those of low quality, and arrays were normalized so that the median of ratio across each array was equal to 1.0. Automatically and manually flagged spots, spots where the sum of median (635/532) signal intensities were ≤ 100 , and spots with signal intensities below the threshold (i.e., the sum of median intensities plus one standard deviation above the mean background) were filtered out prior to analysis. Ratio data from the three biological replicates were compiled and normalized based on the total percent Cy3 intensity and percent Cy5 intensities to eliminate slide-to-slide variation. Gene expression data were then normalized to 16S rRNA. Microarray data has been deposited in ArrayExpress under accession number E-MEXP-1558.

Measurement in vitro growth by determining the optical density. Duplicate mid-log-phase cultures were diluted in Stainer-Scholte broth supplemented with 40 μ g of streptomycin/ml to obtain a starting OD₆₀₀ of 0.02 and grown at 37°C with shaking at 275 rpm. Growth was monitored via measurement of the OD₆₀₀ every 6 h for 48 h.

Bacterial adhesion assays. Rat epithelial (L2) cells and porcine kidney (PK-15) cells were grown to ~80% confluence in a 24-well plate using 50:50 Dulbecco modified Eagle medium-F-12 medium supplemented with 10% fetal bovine serum and then inoculated with bacterial suspensions prepared from KM22, TN27, and TN28 cultures collected in mid-log phase and then diluted to 2.1×10^8 CFU/ml, resulting in a multiplicity of infection of 100 using growth media. Dilutions of bacterial inocula were plated on BG plates containing 40 μ g of streptomycin/ml to determine CFU counts and verify the multiplicity of infection. Adhesion assays were carried out in triplicate and performed by removing growth medium from the L2 cells and PK-15 cells and then adding either medium alone or medium plus 1 ml of each bacterial inoculum. Plates were then centrifuged for 5 min at $250 \times g$, followed by incubation at 37°C with 5% CO₂ for 40 min. Wells were then washed four times with 1 ml of the growth medium to remove nonadherent bacteria. L2 and PK-15 cells were then treated with 0.5 ml of 0.125% trypsin, followed by incubation for 10 min at 37°C. The total volume of each well was brought up to 1 ml with growth medium and homogenized by pipetting. Dilutions were plated on BG plates containing 40 μ g of streptomycin/ml to determine CFU counts, which were then used to calculate the proportion of adherent bacteria, expressed as a percentage of the original inoculum. The results were analyzed for significance using the Student *t* test, and a *P* value of <0.05 was considered significant.

Experimental infection in swine. *B. bronchiseptica* strains KM22, TN27 ($\Delta fhaB$), and TN28 (Δprn) were cultured on BG agar supplemented with 10% sheep blood (BG) at 37°C for 40 h. Suspensions of these cultures with an A_{600} of 0.42 were prepared in phosphate-buffered saline (PBS). This suspension has approximately 2×10^9 CFU/ml, and a 1:2,000 dilution of this suspension was made in PBS for inoculation of the pigs. Cultured dilutions of strains KM22, TN27 ($\Delta fhaB$), and TN28 (Δprn) inocula contained 2.4×10^6 , 3.1×10^6 , and 2.5×10^6 CFU/ml, respectively. One hundred percent of the colonies of both strains appeared to be in the Bvg⁺ phase, based on colony morphology and the presence of hemolysis. Fifty-six pigs were divided into three groups of 16 pigs each and one group of 8 pigs. Pigs were inoculated intranasally at 1 week of age with 1 ml (0.5 ml/nostril) of a bacterial suspension of strain KM22, TN27 ($\Delta fhaB$), TN28 (Δprn) or with 1 ml of sterile PBS, respectively. No *B. bronchiseptica* was isolated from nasal swabs prior to the start of the experiment. All housing, husbandry, and experiments performed with pigs were in accordance with the law and approved by the Institutional Animal Care and Use Committee.

Determination of colonization. To measure colonization of the nasal cavity, nasal swabs were taken from each pig on days 1, 3, and 5 postinoculation and placed into tubes containing 500 μ l of PBS. Nasal washes were performed on days 7, 14, 21, 28, 35, 42, 49, and 56 postinoculation and were performed by injecting 5 ml of PBS into the nasal cavity through one nostril and collecting the

effluent into a beaker. Four pigs from each of the *B. bronchiseptica* inoculated groups and two PBS inoculated pigs were euthanized with an overdose of barbiturate, and necropsies were performed 7, 14, 28, and 56 days postinoculation. At necropsy, nasal washes were performed, and snouts were transected and removed at the level of the first premaxillary tooth and a 1-cm cross-section was cut from the caudal portion of the snout and used for atrophic rhinitis scoring. The trachea was then severed just below the larynx, and the trachea and lungs were removed. A tracheal wash was performed by placing an ~8-cm segment of trachea in a 15-ml centrifuge tube with 5 ml of PBS, followed by vigorous shaking. Lung lavage was performed by filling the lungs with 100 ml of sterile PBS, gently massage, and aspiration; approximately 50 ml of the PBS was recovered. Sections of lung taken for microscopic evaluation were fixed in 10% neutral buffered formalin for 24 h and then placed in 90% ethanol. All sections were routinely processed and embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Serial 10-fold dilutions were made from the PBS solution in the tubes with the nasal swabs after vortex mixing and from the collected PBS from the nasal and tracheal washes and the lung lavage. The number of CFU of *B. bronchiseptica* per ml was determined by plating 100 μ l of the dilutions on duplicate selective blood agar plates containing 20 μ g of penicillin, 10 μ g of amphotericin B, 10 μ g of streptomycin, and 10 μ g of spectinomycin/ml. The lowest level of detection was 10 CFU/ml. Colonies from plates representing each respiratory tract site (nasal cavity, trachea, and lungs) were randomly screened to confirm that recovered colonies were the same as the challenged strain for all *B. bronchiseptica*-inoculated groups. Statistical analyses of the colonization data were performed with SAS software 9.1.3 (SAS Institute, Inc., Cary, NC). A mixed model for repeated measures (PROC MIXED) using the spatial power law for unequally spaced data as the repeated effect (37) was utilized, which accounted for the effects of bacterial strain, the interaction of strains, and the time after infection. Prior to analysis the following covariance structures were tested, and the structures with the lowest scores were used in the final analysis: -2 REML log likelihood, Akaike's information criterion, and the Schwarz's Bayesian information criterion. A *P* value of <0.05 was considered significant.

Pathological evaluation of the lung. At necropsy, an estimate of gross lung involvement was assigned based on the percentage of each lung lobe affected and the percentage of total lung volume each lobe represented. The percentage of total lung volume of each lobe was estimated as 10% for the left cranial, 10% for the left middle, 25% for the left caudal, 10% for the right cranial, 10% for the right middle, 25% for the right caudal, and 10% for the intermediate lung lobes.

Atrophic rhinitis scores. Snouts were transversely sectioned at the level of the first premaxillary tooth, and each of the four scrolls of the ventral turbinate was assigned an atrophy score that as follows: 0, normal; 1, more than half of turbinate remaining; 2, half or less of turbinate remaining; 3, turbinate is straightened with only a small portion left; and 4, total atrophy. The atrophic rhinitis score is the sum of the four turbinate atrophy scores and ranged from 0 to 16.

Antibody response analysis. Sera were collected from infected and control pigs on days 21, 35, and 56 postchallenge. Titers of anti-*Bordetella* antibodies were measured by enzyme-linked immunosorbent assay (ELISA) adapting a method previously described for mice (39). Briefly, plates were coated with heat-killed *B. bronchiseptica* KM22 (grown to an OD₆₀₀ of 0.6). Sera were serially diluted, and *B. bronchiseptica*-specific antibody was detected using secondary antibodies specific for swine immunoglobulin G (IgG) or IgM (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Endpoint antibody titers were expressed as the reciprocal of the dilution giving an OD at 405 nm of 0.1 higher than the average of the optical density measured for negative control after 45-min incubation with substrate. Statistical analyses of the antibody data were performed with SAS software 9.1.3 using PROC MIXED with compound symmetric covariance structure in the repeated statement. The model included the bacterial strain, the time, and the interaction of strain and time. Prior to either analysis the following covariance structures were tested, and the structure with the lowest scores were used in the final analysis: -2 REML log likelihood, Akaike's information criterion, and the Schwarz's Bayesian information criterion. A *P* value of <0.05 was considered significant.

Antibody-complement killing assay. Sera collected on day 56 postinfection from KM22, TN27, and TN28-infected or PBS-treated pigs were heat inactivated by incubation at 56°C for 30 min. Each *B. bronchiseptica* target strain was prepared in the same manner as the bacterial suspensions used for inoculation of the pigs. Briefly, each target strain was cultured on BG plates at 37°C for 40 h. Suspensions of these cultures with an A_{600} of 0.42 were prepared in PBS and diluted to 2×10^6 CFU/ml. Each antibody-complement killing assay was run in triplicate and set up as follows: 10 μ l of swine sera, 5 μ l of guinea pig sera (complement source; Sigma, St. Louis, MO), and 100 μ l of 2×10^6 CFU/ml of target strain were added to a single well of a 96-well round-bottom plate. Stainer-Scholte broth containing 0.06% bovine serum albumin (Sigma) was

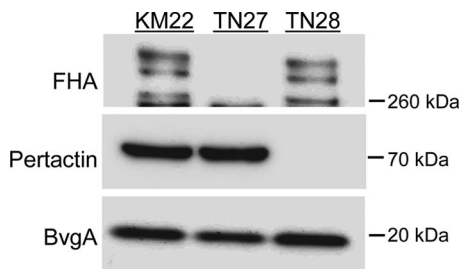


FIG. 1. Western blot analysis of whole-cell bacterial lysates of wild-type *B. bronchiseptica* strain KM22, TN27 ($\Delta fhaB$), and TN28 (Δprn). *B. bronchiseptica* strains were grown to logarithmic phase, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and divided into three sections. Each section was probed for either FHA, PRN, or BvgA. Sizes are indicated in kilodaltons on the right.

added to bring the final volume to 0.3 ml per well. Wells containing infected swine sera only, guinea pig sera only, or bacteria only were included as controls. Plates were then incubated at 37°C for 4 h with gentle agitation. Serial dilutions in PBS were then plated on blood agar plates to determine CFU counts, which were then used to calculate the ratio of bacteria in treatment wells compared to control wells and reported as the percent viable bacteria. The results were analyzed for significance using the Student *t* test, and a *P* value of <0.05 was considered significant.

RESULTS

Construction and characterization of FHA and PRN mutants. To examine the contribution of FHA and PRN in *Bordetella* pathogenesis in swine, we constructed in-frame nonpolar deletions in the *fhaB* gene or the *prn* gene of KM22, a virulent *B. bronchiseptica* swine isolate. Based on exhibiting a shared ribotype and pertactin type as the majority of strains isolated from swine, KM22 was chosen to represent a commonly isolated *B. bronchiseptica* field strain (53–55, 57). In addition, KM22 has been successfully used by our laboratory to generate a reproducible swine respiratory disease model reflective of clinical *B. bronchiseptica* infections among swine herds (8–13, 56). Strain TN27 contains a large in-frame deletion in *fhaB*, the FHA structural gene, and strain TN28 contains a large in-frame deletion in *prn*, the PRN structural gene. Protein expression of these mutant strains was assessed by Western blot analysis. Whole-cell lysates of wild-type *B. bronchiseptica* strain KM22, and mutants TN27 ($\Delta fhaB$) and TN28

(Δprn) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and divided into three sections. Each section was then probed for the presence of FHA, PRN, or BvgA. FHA was detected in strains KM22 and TN28 but not in strain TN27 ($\Delta fhaB$) (Fig. 1). PRN was detected in strains KM22 and TN27 ($\Delta fhaB$) but not in TN28 (Δprn) (Fig. 1). BvgA was detected in all strains (Fig. 1). Using the anti-FHA antibody, a ~240-kDa polypeptide was detected in the whole-cell lysates of all three strains. To ensure that this polypeptide was not a truncated form of FHA, we additionally evaluated all of the strains along with the previously well-characterized strains RB50 and RBX9 (RB50 containing an in-frame deletion of the *fhaB* gene) (16) by Western blot analysis. A similar ~240-kDa polypeptide was also detected in the whole-cell lysates of RBX9 (data not shown), indicating that this polypeptide was not a truncated form of FHA. These data confirm the absence of FHA and PRN in strains TN27 ($\Delta fhaB$) and TN28 (Δprn), respectively.

To ensure that only the transcripts *fhaB* or *prn* were affected in strains TN27 ($\Delta fhaB$) and TN28 (Δprn), respectively, gene expression of these and other selected transcripts were evaluated by quantitative reverse transcription-PCR. Similar expression levels were detected in KM22 and TN27 ($\Delta fhaB$) for *fimA* and *bvgA*, which lie directly upstream and downstream of *fhaB*, the housekeeping gene *pgk*, *bvgS*, *bvgR*, and *fhaC*, which is required for the export of FHA (21, 30, 31, 59) (Fig. 2A). In contrast, *fhaB* expression was detected in KM22 and TN28 (Δprn) and not in TN27 ($\Delta fhaB$) (Fig. 2A). Similar expression levels were also detected in KM22 and TN28 (Δprn) for *upp* and *cysG*, which lie directly upstream and downstream of *prn*, *bvgA*, *bvgS*, *bvgR*, *fhaB*, and *pgk* encoding a housekeeping gene (Fig. 2B). In addition, expression of *prn* was only detected in KM22 and TN27 ($\Delta fhaB$) and not in TN28 (Δprn) (Fig. 2). To globally determine the absence of polar effects in the creation of these strains, whole-transcriptome analysis was performed to determine genes differentially expressed between KM22 and each mutant strain. Of 5,013 open reading frames analyzed, only Bb2993 *fhaB* was upregulated in KM22 relative to TN27 ($\Delta fhaB$) (data not shown), and only Bb1366 *prn* was upregulated in KM22 relative to TN28 (Δprn) (data not shown). Together, these data confirm that only the transcripts *fhaB* or *prn* were affected in strains TN27 ($\Delta fhaB$) and TN28 (Δprn), respectively, and the absence of polar effects in the creation of

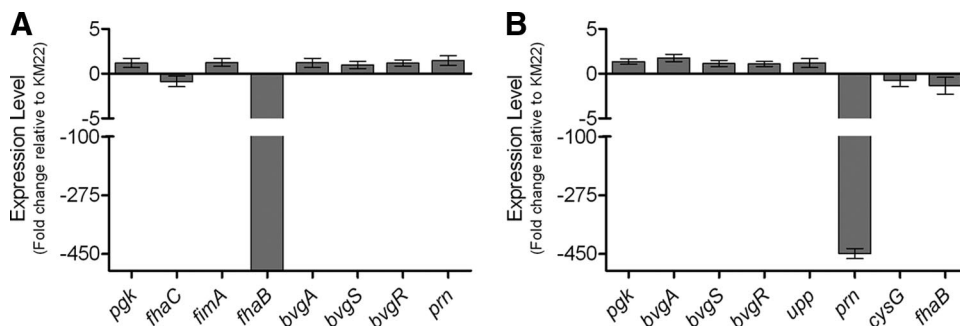


FIG. 2. Gene expression changes in *B. bronchiseptica* strains TN27 (A) and TN28 (B) evaluated by quantitative reverse transcription-PCR. The x axis indicates the genes analyzed. The y axis indicates the fold change in gene expression of each strain relative to KM22 using the relative quantitative method ($\Delta\Delta C_T$). Error bars represent \pm the standard error.

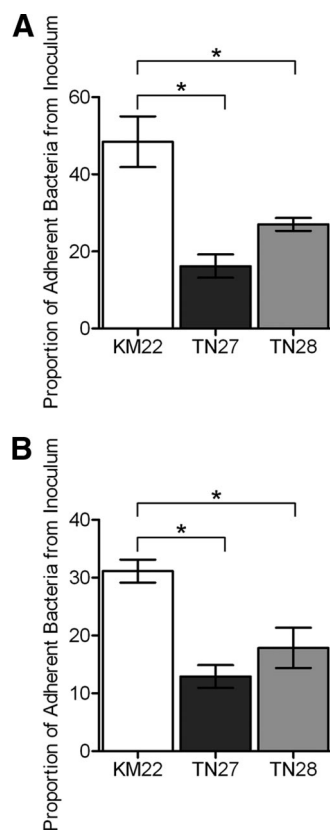


FIG. 3. *in vitro* adherence of *B. bronchiseptica* strains KM22, TN27 ($\Delta fhaB$), and TN28 (Δprn). Adherence of bacteria to L2 cells (A) and PK-15 cells (B) is expressed as the proportion of adherent bacteria to the original inoculum. The error bars represent \pm the standard deviation. An asterisk indicates that the *P* value is <0.05 .

these strains. Next, we monitored the growth rate of each strain *in vitro* to ensure that deletions in the *fhaB* gene and the *prn* gene did not alter the growth rate of these strains. Based on the growth curve data (data not shown), both TN27 ($\Delta fhaB$) and TN28 (Δprn) grew at similar rates as KM22, indicating that deletions in the *fhaB* gene and the *prn* gene did not alter the growth rate of TN27 ($\Delta fhaB$) and TN28 (Δprn) *in vitro*.

***In vitro* adherence of FHA and PRN mutants.** The role of FHA as an adhesin is well documented, and both PRN and FHA have been reported to mediate adherence to various cell lines *in vitro*. Therefore, we hypothesized that both TN27 ($\Delta fhaB$) and TN28 (Δprn) would be defective in the ability to adhere to cell lines *in vitro* compared to KM22. To test this, we compared the ability of TN27 ($\Delta fhaB$) and TN28 (Δprn) to KM22 in mediating adherence in a standard adherence assay. L2 cells were chosen to be evaluated due to routine use in attachment assays involving *Bordetella* (16, 40). Attachment to a porcine epithelial cell line was also included in this assay. Given that a porcine respiratory tract cell line is not available, we chose to use a kidney epithelial cell, PK-15. PK-15 cells are routinely used to propagate and isolate swine respiratory viruses such as porcine respiratory coronavirus (62), which is used as a model for severe acute respiratory syndrome (61, 74). Significantly fewer TN27 ($\Delta fhaB$) and TN28 (Δprn) bacteria adhered to L2 cells than KM22 ($P < 0.05$) (Fig. 3A), suggest-

ing that both FHA and PRN serve a role in mediating *in vitro* adherence for KM22, a *B. bronchiseptica* swine isolate. These results are consistent with previous reports demonstrating a role for FHA in mediating *B. bronchiseptica* adherence to L2 cells (16, 26, 40). However, to our knowledge, the present study is the first report demonstrating that PRN mediates the adherence of *B. bronchiseptica* to L2 cells. Similar to the adherence results involving L2 cells, significantly less TN27 ($\Delta fhaB$) and TN28 (Δprn) adhered to PK-15 cells than did to KM22 ($P < 0.05$) (Fig. 3B). Together, these results demonstrate a role for both FHA and PRN in mediating *in vitro* adherence for KM22, a *B. bronchiseptica* swine isolate.

Clinical response of pigs. To evaluate the role of PRN and FHA in swine respiratory tract infection, we intranasally inoculated groups of 1-week-old piglets with *B. bronchiseptica* strain KM22, TN27 ($\Delta fhaB$), or TN28 (Δprn) or with 1 ml of sterile PBS, respectively. Clinical signs were only noted in piglets from groups inoculated with KM22 or TN28 (Δprn). These signs were generally mild and mainly consisted of sneezing and coughing. No clinical signs were observed in piglets from groups inoculated with TN27 ($\Delta fhaB$) or PBS.

Colonization of the respiratory tract. (i) Nasal cavity. Colonization of the nasal cavity was evaluated on days 1, 3, 5, and 7 and weekly thereafter until day 56 postinfection (Fig. 4A). No *B. bronchiseptica* CFU were recovered from any site in the respiratory tract of piglets inoculated with PBS. During monitoring of the colonization of the nasal cavity, as well as of the trachea and lung, single colonies were randomly screened to confirm that recovered bacteria were the same as the challenge strain for all *B. bronchiseptica* inoculated groups. In general, significantly ($P < 0.0001$) lower CFU levels were recovered from TN27 ($\Delta fhaB$)-inoculated pigs than from pigs infected with either KM22 or TN28 (Δprn). Focusing on specific days, TN27 ($\Delta fhaB$) was recovered at lower CFU levels than KM22 at day 3 and day 5. At day 7, the levels of CFU recovered from TN27 ($\Delta fhaB$)-infected piglets began to approach the levels recovered from KM22-infected piglets and then declined. Lower CFU levels were then recovered on day 14 from TN27 ($\Delta fhaB$)-infected pigs relative to CFU levels recovered from KM22-infected pigs and remained lower for all time points thereafter. At day 35, the levels of CFU recovered from TN27 ($\Delta fhaB$)-infected pigs fell below detectable levels. This was then followed by a small rise in the levels of CFU recovered from TN27 ($\Delta fhaB$)-infected pigs; however, these levels remained lower at all time points relative to the CFU levels recovered from pigs infected with either KM22 or TN28 (Δprn). Taken together, these data suggest that FHA is required for optimal nasal colonization in swine.

On day 1 postinfection, the CFU levels recovered from TN28 (Δprn)-infected pigs were significantly ($P = 0.0006$) lower than the CFU levels recovered from KM22-infected pigs. By day 3 postinfection, the CFU levels recovered from TN28 (Δprn)-infected pigs were similar to the CFU levels recovered from KM22-infected pigs, until day 21 postinfection when the CFU levels recovered from TN28 (Δprn)-infected pigs were less than the levels recovered from KM22-infected pigs. The CFU levels recovered from TN28 (Δprn)-infected pigs were similar to the CFU levels recovered from KM22-infected pigs on day 28 postinfection and remained similar until days 49 and 56 postinfection. At these late time points, the CFU levels

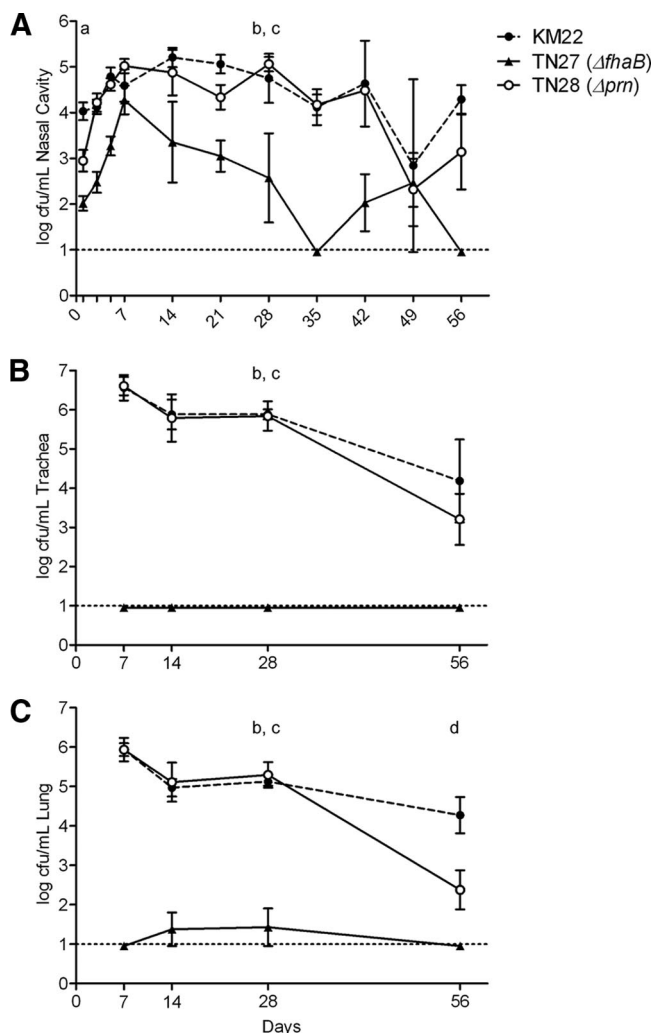


FIG. 4. Colonization of the swine respiratory tract by wild-type *B. bronchiseptica* strain KM22, TN27 ($\Delta fhaB$), and TN28 (Δprn). Groups of 16 pigs were inoculated intranasally with KM22 (●), TN27 (▲), and TN28 (○). (A) The bacterial load in the nasal cavity was quantified 1, 3, 5, 7, 14, 21, 28, 35, 42, 49, and 56 days postinoculation. (B and C) The bacterial load in the trachea (B) and the lungs (C) was quantified 7, 14, 28, and 56 days postinoculation. The x axis indicates days postinoculation, and the y axis indicates the mean CFU expressed as the \log_{10} mean \pm the standard error (error bars). The dashed line indicates the lower limit of detection ($\log_{10}1$). The statistical differences are indicated as $P = 0.0006$ for TN28 compared to KM22 on day 1 postinoculation (a), $P < 0.0001$ for TN27 compared to KM22 assessed as repeated measurements over all time points (b), $P < 0.0001$ for TN27 compared to TN28 assessed as repeated measurements over all time points (c), and $P = 0.0025$ for TN28 compared to KM22 on day 56 postinoculation (d). A P value less than 0.05 was considered significant.

recovered from TN28 (Δprn)-infected pigs were lower than the CFU levels recovered from KM22-infected pigs. The significantly lower CFU levels of TN28 (Δprn) relative to KM22 recovered on day 1 postinfection suggest that PRN aids in early attachment of the swine nasal cavity.

(ii) **Trachea.** Colonization of the trachea was evaluated on days 7, 14, 28, and 56 postinfection (Fig. 4B). *B. bronchiseptica* was never recovered from the tracheas of any pigs infected with TN27 ($\Delta fhaB$), and thus the CFU levels recovered from

TN27 ($\Delta fhaB$)-inoculated pigs were significantly ($P < 0.0001$) lower than the CFU levels recovered from pigs infected with either KM22 or TN28 (Δprn). These data suggest that FHA is required for colonization of the trachea in swine and are consistent with previous results (16). In contrast, TN28 (Δprn) was recovered at similar levels as KM22 until day 56 postinfection. At this time TN28 (Δprn) was recovered at a lower level than KM22.

(iii) **Lung.** Colonization of the lungs was evaluated on days 7, 14, 28, and 56 postinfection (Fig. 4C). In general, significantly ($P < 0.0001$) lower CFU levels were recovered from the lungs of TN27 ($\Delta fhaB$)-inoculated pigs than from the lungs of pigs infected with either KM22 or TN28 (Δprn). When evaluating lung colonization on specific days, we recovered undetectable CFU levels from TN27 ($\Delta fhaB$)-infected pigs on days 7 and 56 postinfection and, unexpectedly, we recovered low levels of TN27 ($\Delta fhaB$) in one of four pigs on days 14 and 28 postinfection. Together, these data suggest that FHA is required for lung colonization in swine and is consistent with previous results (16). The CFU levels recovered from the lungs of TN28 (Δprn)-infected pigs were similar to levels recovered from KM22-infected pigs until day 56 postinfection. At this late time point, the CFU levels recovered from the lungs of TN28 (Δprn)-infected pigs were significantly ($P = 0.0025$) lower than the CFU levels recovered from the lungs of KM22-infected pigs. This decreased colonization level suggests a role for PRN in persistence of the lower respiratory tract in swine.

Pathology. (i) Turbinate. The degree of atrophic rhinitis was evaluated by determining the mean turbinate atrophy score from piglets inoculated with KM22, TN27 ($\Delta fhaB$), TN28 (Δprn), or PBS on days 7, 14, 28, and 56 postinfection. No significant degree of turbinate atrophy was observed from pigs infected with PBS or TN27 ($\Delta fhaB$) (Table 1). Only piglets infected with either KM22 or TN28 (Δprn) had any evidence of significant nasal turbinate atrophy, a finding reflective of an

TABLE 1. Mean turbinate atrophy scores and percentages of the lungs affected by pneumonia in pigs inoculated with *B. bronchiseptica* strains KM22, TN27 ($\Delta fhaB$), TN28 (Δprn), or PBS

Day of necropsy	Experimental group	Mean turbinate atrophy score (range)	Mean % of pneumonia (range)
7	PBS control	0.5 (0–1)	0 (0)
	KM22	2 (0–4)	6.5 (0–18)
	TN27 ($\Delta fhaB$)	0 (0)	0 (0)
	TN28 (Δprn)	2 (0–4)	2.25 (0–4)
14	PBS control	0 (0)	0 (0)
	KM22	3.33 (0–8)	1.67 (1–2)
	TN27 ($\Delta fhaB$)	0.5 (0–2)	0 (0)
	TN28 (Δprn)	3 (0–8)	2.25 (0–4)
28	PBS control	1 (1)	0 (0)
	KM22	2.67 (0–6)	0 (0)
	TN27 ($\Delta fhaB$)	0.25 (0–1)	0 (0)
	TN28 (Δprn)	2.5 (0–5)	6.25 (0–25)
56	PBS control	0.5 (0–1)	0 (0)
	KM22	2.5 (1–4)	6.75 (4–9.5)
	TN27 ($\Delta fhaB$)	0.75 (0–2)	0 (0)
	TN28 (Δprn)	3 (2–6)	5.25 (3–11)

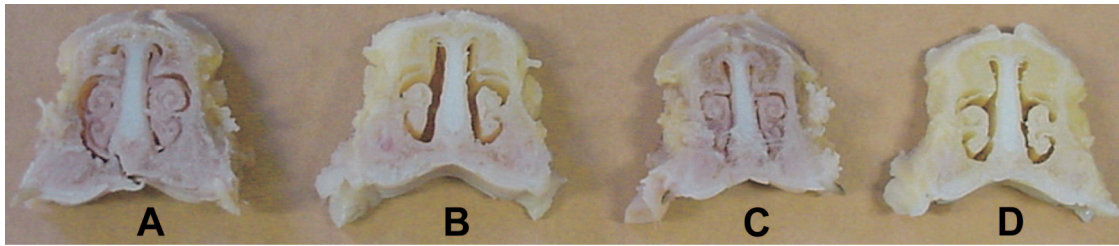


FIG. 5. Cross-section of snouts showing the degree of turbinate atrophy at 14 days postinoculation in pigs inoculated with PBS (A), KM22 (B), TN27 ($\Delta fhaB$) (C), or TN28 (Δprm) (D).

atrophic rhinitis score greater than 2 (Table 1). The increased degree of turbinate atrophy observed in KM22- and TN28 (Δprm)-inoculated pigs relative to TN27 ($\Delta fhaB$)- or PBS-inoculated infected pigs is shown in the cross-sections of snouts taken at 14 days postinfection (Fig. 5).

(ii) **Lung.** Pathological evaluation of the lung was assessed by determining the mean percentage of lung affected by pneumonia in piglets inoculated with KM22, TN27 ($\Delta fhaB$), TN28 (Δprm), or PBS on days 7, 14, 28, and 56 postinfection. Overall, no pneumonia was observed in TN27 ($\Delta fhaB$)- or PBS-inoculated piglets, whereas a similar degree of pneumonia was observed in TN28 (Δprm)- and KM22-inoculated pigs. The pneumonia exhibited in TN28 (Δprm)- and KM22-infected pigs consisted of areas of red, seen 7 and 14 days postinfection, to tan, seen 28 and 56 days postinfection, consolidation with well-demarcated borders and a cranial ventral distribution. Microscopically, the lesions in both KM22 and TN28 (Δprm)-challenged pigs were typical of *B. bronchiseptica* pneumonia, characterized by suppurative bronchopneumonia, which is gradually replaced by fibroplasia. For KM22- and TN28 (Δprm)-challenged pigs, the percentage of lung affected ranged from 0 to 18% for pigs infected with KM22 and 0 to 25% for pigs infected with TN28 (Δprm) (Table 1). On day 28 postinfection, despite equivalent bacteria recovered from the lungs, the mean percentage of lung affected by pneumonia in TN28 (Δprm)-challenged pigs was 6.25, and no pneumonia was observed in KM22-infected pigs (Table 1). Overall, 64% of the pigs infected with KM22 and 69% of the pigs infected with TN28 (Δprm) exhibited pneumonia. Thus, the difference in pneumonia observed at day 28 between KM22- and TN28 (Δprm)-challenged pigs likely reflects a stochastic event rather than a pathology trend.

Antibody response. To investigate the role of FHA and PRN in the development of anti-*Bordetella* humoral immunity, the serum levels of anti-*Bordetella* IgM (Fig. 6A) and IgG (Fig. 6B) were quantified in pigs inoculated with KM22, TN27 ($\Delta fhaB$), TN28 (Δprm), or PBS by ELISA using heat-killed KM22 whole cells as the antigen. The data represent samples collected from the same pigs throughout the experiment, and the levels of anti-*Bordetella* IgM and IgG were below the limit of detection in PBS-inoculated pigs (data not shown). Similar levels of IgM were detected in KM22-, TN27 ($\Delta fhaB$)-, and TN28 (Δprm)-infected pigs on day 21 postinfection (Fig. 6A). On day 35 postinfection, higher ($P = 0.07$) levels of IgM were detected in TN28 (Δprm)-infected pigs compared to IgM levels for KM22- and TN27 ($\Delta fhaB$)-infected pigs (Fig. 6A). Significantly ($P \leq 0.014$) higher levels of IgM were detected in KM22-infected

pigs on day 56 compared to IgM levels from TN27 ($\Delta fhaB$)- and TN28 (Δprm)-infected pigs. Although IgM levels were significantly higher in KM22-infected pigs at 56 days postinfection, the serum IgM levels did not significantly differ over time in either KM22- or TN27 ($\Delta fhaB$)-challenged animals.

In general, significantly ($P = 0.0059$) higher serum anti-*Bordetella* IgG levels were detected in TN28 (Δprm)-infected pigs compared to KM22- and TN27 ($\Delta fhaB$)-infected pigs (Fig. 6B). Focusing on specific days, similar anti-*Bordetella* IgG lev-

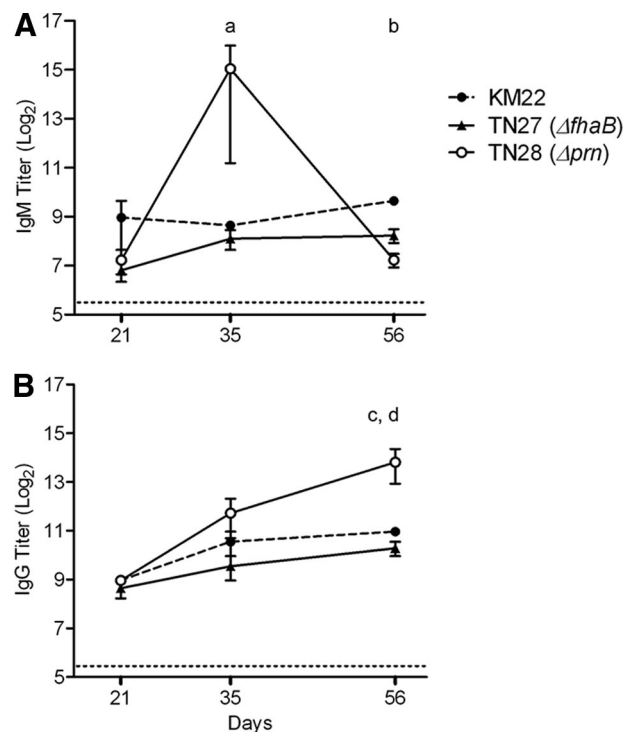


FIG. 6. Serum anti-*Bordetella* IgM (A) and IgG (B) titers. Sera were collected from the same pigs within infected and control groups on days 21, 35, and 56 postchallenge. Titers of anti-*Bordetella* antibodies were measured by ELISA using *B. bronchiseptica* KM22 as the antigen. The x axis indicates days postinoculation, and the y axis indicates the \log_2 mean relative titer \pm the standard error (error bars). The dashed line indicates the lower limit of detection (50 or $\log_2 5.4$). Statistical differences are indicated as $P = 0.07$ for TN28 compared to KM22 (a), $P \leq 0.014$ for KM22 compared to TN28 and TN27 (b), $P = 0.0059$ for TN28 compared to KM22 and TN27 assessed as repeated measurements over all time points (c), and $P = 0.057$ for TN28 compared to KM22 on day 56 postinoculation (d). A P value of <0.05 was considered significant.

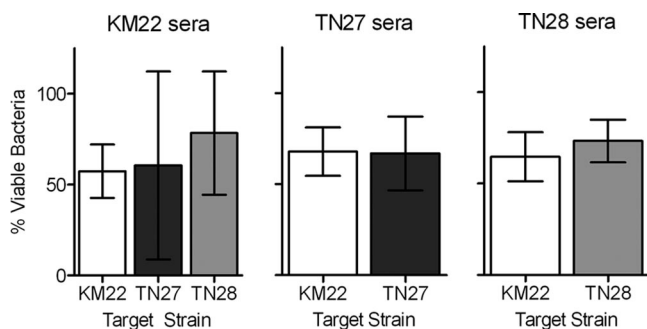


FIG. 7. Bacterial killing by immune serum-complement mediated lysis. Sera collected on day 56 postchallenge from pigs inoculated with KM22, TN27, TN28, or PBS were incubated with a target strain of *B. bronchiseptica* (x axis) in the presence of guinea pig sera (complement source) as described in Materials and Methods. After incubation, bacteria were enumerated to determine the percent viable bacteria (y axis) expressed as the mean \pm the standard error (error bars). A *P* value of <0.05 was considered significant.

els were detected in KM22-, TN27 ($\Delta fhaB$)-, and TN28 (Δprn)-infected pigs on days 21 and 35 postinfection (Fig. 6B). On day 56 postinfection, higher ($P = 0.057$) levels of IgG were detected in TN28 (Δprn)-infected pigs than in KM22- and TN27 ($\Delta fhaB$)-infected pigs (Fig. 6B). These results suggest that despite a decreased level of colonization throughout the respiratory tract by TN27 ($\Delta fhaB$), this mutant induced a serum IgM and IgG response similar to that induced by wild-type KM22. In addition, these data demonstrate that the absence of PRN results in higher serum anti-*Bordetella* antibody titers during respiratory disease in swine, given that higher serum IgM and IgG levels were detected in TN28 (Δprn)-infected pigs.

Due to the elevated serum anti-*Bordetella* antibody titers detected in TN28 (Δprn)-infected pigs, we determined whether the antibodies elicited by either TN27 ($\Delta fhaB$) or TN28 (Δprn) were more or less bactericidal than the antibodies elicited by KM22. Sera collected on day 56 postinfection from KM22-, TN27-, TN28-, and PBS-inoculated pigs were used in an antibody-complement killing assays, along with *B. bronchiseptica* target strains. Sera from KM22-infected pigs induced similar levels of KM22, TN27 ($\Delta fhaB$), and TN28 (Δprn) killing (Fig. 7). Likewise, sera from TN27 ($\Delta fhaB$)-challenged pigs induced similar levels of killing of KM22 and TN27 ($\Delta fhaB$), and sera from TN28 (Δprn)-infected pigs resulted in equal killing of KM22 and TN28 (Δprn) (Fig. 7). Sera from PBS-inoculated pigs (naive) did not exhibit any antibody-complement killing (data not shown). Taken together, these results show that the antibodies elicited by either TN27 ($\Delta fhaB$) or TN28 (Δprn) were as bactericidal as the antibodies elicited by KM22. In addition, sera from pigs infected with mutated strains of *B. bronchiseptica* induced equal killing of the wild type and the respective mutant strain with which the pigs were infected.

DISCUSSION

Despite widespread use of *B. bronchiseptica* vaccines by swine producers throughout the world, *Bordetella*-associated respiratory diseases remains a significant problem for the industry (22, 68). The development of vaccines with improved efficiency is hindered by the absence of definitive data related

to virulence mechanisms of *B. bronchiseptica* in swine. Previous investigations using rodent and/or rabbit model systems have led to the identification of key aspects in the pathogenesis of *B. bronchiseptica* and have defined protective features of the immune response in these hosts (1, 15, 16, 24, 39, 40). Such information can only be tentatively applied to swine respiratory disease because of basic differences in the structure and organization of the respiratory tract among these animals. For instance, primates are the only animals known to share the same anatomical structure of lymphoid tissues in the upper respiratory tract with pigs (48, 51). In addition, rodents and rabbits also lack the pharyngeal and palatine tonsils that are known to function as inductive sites for secretory antibody responses in swine (34, 73). Evaluation of *B. bronchiseptica* specific virulence factors in swine provides data directly relevant and necessary for designing improved vaccines and therapeutic interventions.

To begin evaluating the role of FHA and PRN in *Bordetella* pathogenesis in swine, we constructed mutants containing an in-frame deletion of the FHA or the PRN structural gene in a virulent *B. bronchiseptica* swine isolate and compared both of these mutants to a wild-type swine isolate for their ability to adhere to L2 and PK-15 cells in a standard adhesion assay. We found that both the FHA and the PRN mutants to be defective in their ability to adhere to both cell lines, demonstrating a role for both FHA and PRN in mediating in vitro adherence for KM22, a *B. bronchiseptica* swine isolate.

Using a swine respiratory disease model, we then compared FHA and PRN mutants to a wild-type swine isolate for their ability to colonize and cause disease. Colonization of the PRN mutant was similar to wild-type except for two time points, early at day 1 and late at day 56 postinfection. The decreased colonization observed at day 1 postinfection was observed in the nasal cavity and suggests that PRN may aid in early attachment. As we monitored colonization levels throughout the infection, the PRN mutant was recovered at similar levels as wild-type until day 56 postinfection. At this last time point, the wild-type isolate, KM22, was recovered at more than a log-fold higher level than the PRN mutant in both the nasal cavity and the trachea, and two log-fold higher than the PRN mutant in the lung. The decreased colonization levels of the PRN mutant at the last time point implies a role for PRN in persistence in the swine respiratory tract. However, since the colonization data presented here did not continue until clearance, a definitive role for PRN in the persistence of the swine respiratory tract remains unclear. In terms of disease severity, we found clinical signs of disease, such as sneezing and coughing, and pathology, such as turbinate atrophy, to be indistinguishable between pigs infected with the PRN mutant or the wild-type isolate. Thus, PRN appears to play no apparent role in clinical signs or pathology. When anti-*Bordetella* antibodies were quantified, higher serum IgM and IgG levels were detected in pigs infected with the PRN mutant than in pigs infected with either the wild-type swine isolate or the FHA mutant. It has previously been demonstrated that anti-*Bordetella* antibodies aid in the clearance of *B. bronchiseptica* from the lower respiratory tract (50). Therefore, the higher anti-*Bordetella* antibodies induced by the PRN mutant may have contributed to the decreased bacterial burdens in the lung at this late infection time point. Both the decreased in vitro adherence and decreased

colonization at day 1 postinfection by the PRN mutant demonstrates a role for PRN in mediating adherence. Thus, the decreased bacterial burdens by the PRN mutant observed late in the infection may alternatively be attributed to the role of PRN in mediating attachment.

In contrast to the PRN mutant, we found a significant defect in the ability of the FHA mutant to colonize throughout the respiratory tract, including the nasal cavity. In following colonization of the nasal cavity, the FHA mutant was recovered at lower levels compared to both the wild-type and the PRN mutant for every time point analyzed. When we monitored colonization of the trachea and the lungs, no CFU were recovered from the trachea of any pig infected with the FHA mutant at any time, whereas a small number of CFU were detected in the lungs from two pigs within this group. A previous study using a *B. bronchiseptica* rabbit isolate, containing a similar in-frame deletion in the FHA structural gene, demonstrated that FHA is required for colonization of the trachea in a rat respiratory infection model (16). The trachea and lung colonization data in this report are consistent with these previous findings and further contribute to the conclusion put forth by Cotter et al. that FHA is absolutely required for tracheal colonization (16). In the same study, Cotter et al. also demonstrated that FHA is not required for colonization of the nasal cavity (16). The colonization data presented here showing a defect in the ability of the FHA mutant to colonize the swine nasal cavity to wild-type levels are, however, not consistent with this report. One possible explanation for this difference is the *B. bronchiseptica* isolate and animal model used. Focusing on the *B. bronchiseptica* isolate, unlike RB50, which has been sequenced (47), KM22 has not been sequenced so it is possible that KM22 is missing some accessory factor(s) present in RB50, which contributes to nasal colonization along with FHA. Focusing on the animal model used, the previous studies used a *B. bronchiseptica* rabbit isolate in a rat infection model, whereas we used a *B. bronchiseptica* swine isolate within a swine infection model. Recently, a report demonstrated that FHA plays a role in host specificity (26), which may explain the different phenotypes observed between the different infection models. Thus, in some instances it may be beneficial to use an infection system that utilizes an isolate and its natural host when evaluating the role of specific *B. bronchiseptica* virulence factors in pathogenesis.

When anti-*Bordetella* antibodies were quantified, similar levels were detected in pigs infected with either the wild-type swine isolate or the FHA mutant. This was surprising given that the FHA mutant failed to colonize the nasal cavity to levels comparable to the levels of wild-type and/or the PRN mutant. In addition, the FHA mutant was not isolated from the trachea and was only isolated from the lungs of two animals. Although low CFU levels were recovered from these infected pigs, these data suggest that very low levels of *B. bronchiseptica* are required in the nasal cavity to generate anti-*Bordetella* antibodies in swine. Nonetheless, the levels of anti-*Bordetella* antibodies generated in wild type-challenged pigs did not increase significantly between days 21 and 56 postinfection, although bacteria were recovered from all respiratory tract sites. The lack of an increased antibody response overtime may explain the inability of pigs to clear wild-type *B. bronchiseptica* from the lower respiratory tract, since serum-derived antibody

has been shown to be involved in clearance of *Bordetella* from the lower respiratory tract (50).

Higher serum IgM and IgG levels were detected in pigs infected with the PRN mutant compared to the levels of anti-*Bordetella* antibodies generated in wild type- or FHA mutant-challenged pigs. Sera collected on day 56 postinfection from all *Bordetella* challenge groups were equally effective at inducing complement-killing of the respective strain in which the pig was infected, as well as wild-type KM22. This indicates that the antibody generated after infection with the FHA or PRN mutant did not exhibit increased bactericidal effects. Infection with the PRN mutant simply induced higher anti-*Bordetella* immunoglobulin levels. Taken together, these data suggest that PRN may be involved in hindering the humoral antibody response to *Bordetella*. Alternatively, it is possible that the PRN protein may shield other surface antigens or epitopes from immune recognition in wild-type bacteria. The lack of PRN protein in the PRN mutant could also result in architectural changes to the bacterial cell surface; thus, when pigs were infected with the PRN mutant, new antigens were then accessible to the swine immune system. Further studies are warranted to explore these options and determine whether PRN shields immunogenic epitopes or whether PRN can directly alter the production of immunoglobulin.

In terms of disease severity and pathology, we found no differences between pigs infected with the FHA mutant and PBS-inoculated pigs. This was, again, surprising given that despite the major defect in the ability of FHA mutant to colonize the nasal cavity, compared to the wild type and the PRN mutant, CFU were recovered, albeit at lower levels, from these infected pigs. The lack of disease presentation in pigs infected with the FHA mutant suggests that a critical colonization level is required to cause clinical signs of disease, such as sneezing and coughing, and pathology, such as turbinate atrophy.

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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