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The O Antigen Enables *Bordetella parapertussis* To Avoid *Bordetella pertussis*-Induced Immunity▽

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***Bordetella pertussis* and *Bordetella parapertussis* are closely related endemic human pathogens which cause whooping cough, a disease that is reemerging in human populations. Despite how closely related these pathogens are, their coexistence and the limited efficacy of *B. pertussis* vaccines against *B. parapertussis* suggest a lack of cross-protective immunity between the two. We sought to address the ability of infection-induced immunity against one of these pathogens to protect against subsequent infection by the other using a mouse model of infection. Immunity induced by *B. parapertussis* infection protected against subsequent infections by either species. However, immunity induced by *B. pertussis* infection prevented subsequent *B. pertussis* infections but did not protect against *B. parapertussis* infections. The O antigen of *B. parapertussis* inhibited binding of antibodies to the bacterial surface and was required for *B. parapertussis* to colonize mice convalescent from *B. pertussis* infection. Thus, the O antigen of *B. parapertussis* confers asymmetrical cross-immunity between the causative agents of whooping cough. We propose that these findings warrant investigation of the relative role of *B. parapertussis* in the resurgence of whooping cough.**

Bordetella pertussis and *Bordetella parapertussis* are the causative agents of whooping cough (21, 37), a disease that is endemic worldwide despite extensive vaccination efforts (48). Upon the introduction of *B. pertussis* vaccines more than 50 years ago, the incidence of whooping cough greatly declined in developed countries (9). However, the incidence of this disease has been steadily rising over the past 10 to 20 years in populations despite excellent vaccine coverage (7, 8, 12, 49, 56).

Although disease caused by *B. parapertussis* may be less severe than that caused by *B. pertussis*, infections by these bacteria are clinically indistinguishable except for an acute lymphocytosis that is observed only upon infection of naive hosts by *B. pertussis* (3, 21, 36, 41). Differentiating between these pathogens requires costly laboratory work that does not affect treatment decisions (37), making differential diagnoses rare. When specifically investigated, *B. parapertussis* has been found to cause anywhere from less than 1 percent to greater than 95 percent of whooping cough cases (reviewed in reference 59). Thus, it is unclear how many of the estimated 50 million annual cases of whooping cough (10) may be due to *B. parapertussis* and whether or not this pathogen is contributing to the resurgence of this disease.

The coexistence of these two species in human populations creates a paradox to the ecological theory that two closely related immunizing pathogens cannot occupy the same host population if immunity is cross-protective (1, 14, 16, 30). Because *B. pertussis* and *B. parapertussis* evolved from a common progenitor, *Bordetella bronchiseptica*, and share the majority of their virulence factors (42), cross-immunity could be expected. Furthermore, genetic comparisons of *B. pertussis* and *B. parapertussis* to *B. bronchiseptica* suggest that *B. parapertussis* emerged as a pathogen more recently than *B. pertussis* (28, 35, 42). Since the mean age at the time of primary *B. pertussis* infection for unvaccinated children is less than 5 years (9) and infection-induced immunity is protective 5 to 10 years (60), it is likely that *B. parapertussis* successfully emerged in populations that had some level of immunity to *B. pertussis* (4).

Although adapted to humans, both organisms efficiently colonize and rapidly grow throughout the murine respiratory tract but are ultimately eliminated from the lower respiratory tract by B- and T-cell-dependent immunity (24, 25, 27, 62). Watanabe and Nagai suggested that an immune response induced by one species conferred efficient protection against infections by either species (58). However, bacterial numbers were quantified only 2 weeks postinoculation, when bacterial numbers were significantly reduced even in the respiratory tracts of naive hosts (25, 58). Additionally, the strain used in that study (18-323) is quite distinct from other *B. pertussis* strains, as shown by multilocus enzyme electrophoresis and multilocus sequence typing (13, 55), and may actually be more closely related to *B. bronchiseptica* and *B. parapertussis* strains than other *B. pertussis* strains (2, 40). These problems, along with clinical studies showing that *B. pertussis* vaccines poorly protect against *B. parapertussis* and that these two organisms coexist in the same populations, led us to investigate cross-protective immunity between these two pathogens.

We sought to determine whether or not *B. pertussis* and *B. parapertussis* induced effective cross-immunity using the sequenced prototype strains Tohama I (*B. pertussis*) (42) and 12822

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(*B. parapertussis*) (42), which are indistinguishable from current isolates by multilocus enzyme electrophoresis and multilocus sequence typing (13, 55). *B. parapertussis*-induced immunity protected against both species; however, *B. pertussis*-induced immunity protected only against *B. pertussis* infection. The asymmetrical cross-immunity appeared to be the result of inefficient binding of *B. pertussis*-induced antibodies to *B. parapertussis*. O antigen prevented *B. pertussis*-induced antibodies from binding to *B. parapertussis* in vitro and from clearing the bacteria from the respiratory tract in vivo. Together these data provide a molecular basis for the ability of *B. parapertussis* to avoid *B. pertussis*-induced immunity, an ability that may be critical to the coexistence of these pathogens in human populations.

MATERIALS AND METHODS

Bacterial strains and growth. *B. pertussis* strains 536 and 536_{NaI} (from Duncan Maskell, University of Cambridge, United Kingdom) are streptomycin- and nalidixic acid-resistant derivatives of Tohama I, respectively (51). *B. parapertussis* strain 12822 has been described previously (20), and 12822G is a gentamicin-resistant derivative of the parent strain (62). *B. parapertussis* strains CN2591 (wild type) and CN2591Δ*wbm* (O antigen deficient) have previously been described (44). All were maintained on Bordet-Gengou agar (Difco) containing 10% defibrinated sheep blood (Hema Resources) and appropriate antibiotics. Liquid-culture bacteria were grown at 37°C overnight on a roller drum to mid-log phase in Stainer-Scholte broth.

Animal experiments. C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and bred in our *Bordetella*-free, specific-pathogen-free breeding rooms at The Pennsylvania State University. Four- to 6-week-old mice were sedated with 5% isoflurane (Abbott Laboratories) in oxygen and inoculated by pipetting 50 μl of phosphate-buffered saline (PBS) containing 5×10^5 CFU onto the external nares (25). For challenge experiments, mice were inoculated with 5×10^5 CFU of antibiotic-resistant strains at 28 or 70 days postinoculation. For passive transfer of immune serum, 200 μl of sera from naive or convalescent mice (collected 28 days postinoculation) was intraperitoneally injected just prior to inoculation. All protocols were reviewed by the university IACUC, and all animals were handled in accordance with institutional guidelines.

Bacterial quantification. Mice were sacrificed immediately after inoculation or 3 days postinoculation to quantify bacteria in the nasal cavities, tracheae, and lungs. Tissues were homogenized in PBS and plated at specific dilutions onto Bordet-Gengou agar containing appropriate antibiotics, and colonies were counted 4 days later. The lower limit of detection was 10 CFU (the lower limit of the *y* axes).

Splenocyte restimulations. Splenocytes were isolated by homogenizing spleens through a wire sieve, spinning at 1,500 rpm for 5 min at 4°C, lysing the red blood cells, and then washing the cells with Dulbecco's modified Eagle cell culture medium (HyClone, Logan, UT). Cells (2×10^6) were resuspended in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (HyClone), 1 mM sodium pyruvate (HyClone), 100 μg/ml penicillin and streptomycin (HyClone), and 0.005% β-mercaptoethanol and placed into wells of a 96-well plate. Splenocytes were stimulated with medium or 10^7 heat-killed *B. pertussis* or *B. parapertussis* cells. After 3 days, the supernatant was collected and analyzed for gamma interferon (IFN-γ) and interleukin-10 production as specified by the manufacturers of enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN).

ELISAs. Bacteria were grown to an optical density of 0.7, heat inactivated, diluted in carbonate buffer, and used to coat 96-well plates. Plates were stored for 1 to 4 weeks at 4°C (wells filled with PBS containing Tween 20 plus 1% bovine serum albumin) before use. For ELISAs using live bacteria, bacteria were grown to an optical density of 0.7, diluted in carbonate buffer, added to each well of a 96-well plate, and incubated for 1 h at 37°C prior to use. A 1:25 dilution (for heat-inactivated bacteria) or a 1:10 dilution (for live bacteria) of serum samples was added to the first wells and serially diluted across the plates. Plates were incubated for 2 h at 37°C in a humidified chamber and washed, and goat anti-mouse (immunoglobulin heavy plus light chains) horseradish peroxidase-conjugated antibodies (Southern Biotech, Birmingham, AL) were added. Plates were incubated at 37°C in a humidified chamber for 1 h and washed, and 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) in a phospho-citrate buffer and hydrogen peroxide were added to wells, which were incubated at room temperature in the dark for 30 min and read on a plate reader at 405 nm.

Western blots. Westerns blotting was performed on lysates containing 1×10^8 CFU of heat-killed *B. pertussis* or *B. parapertussis*. Lysates were run on 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels in denaturing conditions. Membranes were probed with serum from *B. pertussis*- or *B. parapertussis*-infected mice at a 1:100 dilution and goat anti-mouse (immunoglobulin heavy plus light chains) horseradish peroxidase-conjugated antibody (Southern Biotech, Birmingham, AL) (at a dilution of 1:15,000) as the detector antibody. The membrane was visualized with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ).

Statistical analysis. The means ± standard deviations (error bars) were determined for CFU, IFN-γ production, and antibody titers. Two-tailed, unpaired Student *t* tests were used to determine statistical significance between groups. Results were also analyzed by nonparametric Mann-Whitney tests with similar significance. All experiments were performed at least twice with similar results.

RESULTS

Cross-immunity between *B. pertussis* and *B. parapertussis* is asymmetrical. The coexistence of *B. pertussis* and *B. parapertussis* in humans led us to assess the ability of these pathogens to induce cross-protective immunity. Although considered human-adapted pathogens, both species efficiently colonize the murine respiratory tract (17, 18) and induce sterilizing protective immunity (25), allowing us to test cross-protection in a mouse model. Mice were left uninfected or inoculated with *B. pertussis* or *B. parapertussis*. Twenty-eight days later, when strong immune responses had been generated and bacteria had been reduced to less than 1,000 CFU/lung (25, 62), these mice were challenged with antibiotic-resistant strains of *B. pertussis* or *B. parapertussis* and sacrificed 3 days later. In comparison to naive mice, mice convalescing from *B. pertussis* infection (*B. pertussis*-convalescent mice) harbored antibiotic-resistant *B. pertussis* numbers that were approximately 20-fold lower in the nasal cavity, 1,000-fold lower in the trachea, and more than 10,000-fold lower in the lungs at 3 days postchallenge (Fig. 1A to C). An immune response to *B. parapertussis* also conferred effective protection against *B. pertussis*, as bacterial numbers were approximately 100-fold lower in the nasal cavities, 10,000-fold lower in the tracheae, and 100,000-fold lower in the lungs of *B. parapertussis*-convalescent mice than in those of naive mice (Fig. 1A to C). Groups of mice were also sacrificed 10 min after inoculation to determine the amount of bacteria delivered by the inoculation, allowing us to quantify the growth or reduction in bacterial numbers from the initial amount deposited in the respiratory tract to the numbers 3 days postinoculation. *B. pertussis* increased in numbers in the nasal cavities ($+10^{0.8}$ CFU), tracheae ($+10^{0.3}$ CFU), and lungs ($+10^{1.6}$ CFU) of naive mice (Fig. 1D to F). Numbers were reduced in the nasal cavities, tracheae, and lungs of *B. pertussis*-immune ($-10^{0.2}$, $-10^{3.0}$, and $-10^{2.2}$ CFU, respectively) and *B. parapertussis*-immune ($-10^{0.3}$, $-10^{2.3}$, and $-10^{3.4}$ CFU, respectively) mice (Fig. 1D to F).

Upon investigating the ability of *B. parapertussis* to colonize immunized hosts, it was found that bacterial numbers were approximately 100-fold lower in the nasal cavities and 100,000-fold lower in the tracheae and lungs of *B. parapertussis*-convalescent mice than in those of naive mice (Fig. 2A to C). However, *B. parapertussis* numbers were only 5-fold lower in the nasal cavities and approximately 20-fold lower in the tracheae and lungs (Fig. 2A to C) of *B. pertussis*-convalescent mice than in those of naive mice. *B. parapertussis* numbers grew over the first 3 days in the nasal cavities ($+10^{1.8}$ CFU), tracheae ($+10^{2.2}$

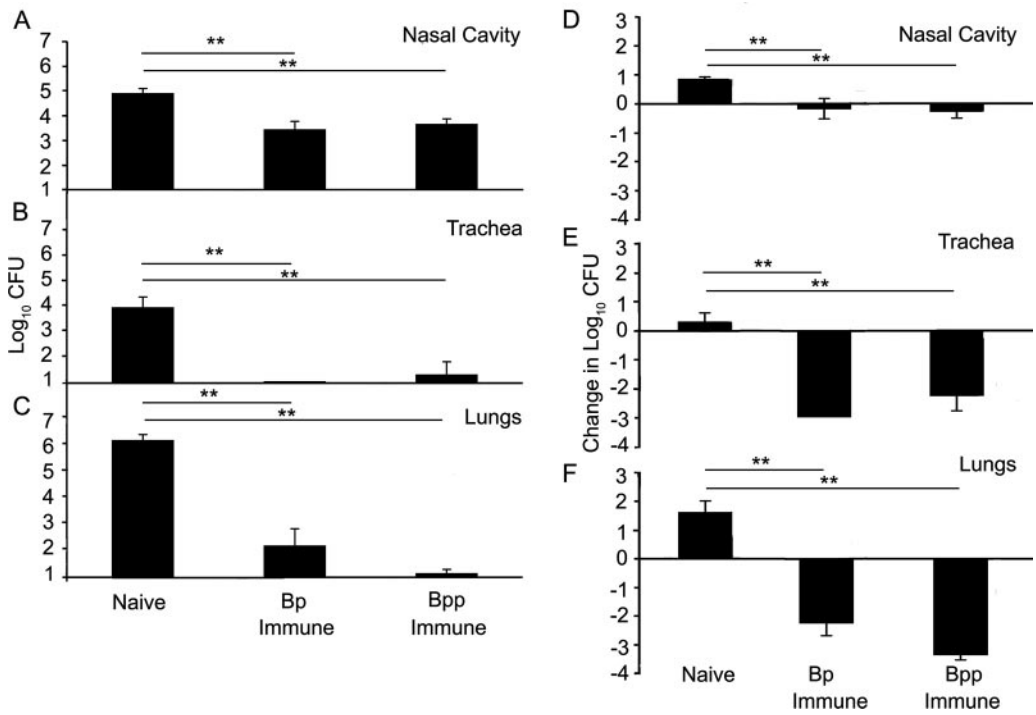

FIG. 1. *B. pertussis* colonization of naive and immunized mice. C57BL/6 mice were inoculated with 5×10^5 CFU of *B. pertussis* (Bp) or *B. parapertussis* (Bpp). Immunized and naive mice were challenged 28 days later with 5×10^5 CFU of a nalidixic acid-resistant strain of *B. pertussis* and sacrificed 3 days after secondary inoculation for the quantification of bacterial numbers in the nasal cavities (A), tracheae (B), and lungs (C). Values are expressed as the $\log_{10}$ means ± standard deviations (SD). For day 3 colonization levels, the difference between each individual value and the mean $\log_{10}$ CFU approximately 10 min after inoculation in the nasal cavities (D), tracheae (E), and lungs (F) was calculated. These data are represented as the mean changes in $\log_{10}$ CFU ± SD. *, $P < 0.05$; **, $P < 0.01$ (compared to naive mice).

CFU), and lungs ($+10^{1.9}$ CFU) of naive mice (Fig. 2D to F). Bacterial numbers were reduced in the nasal cavities, tracheae, and lungs of *B. parapertussis*-immune mice ($-10^{0.4}$, $-10^{2.5}$, and $-10^{3.3}$ CFU, respectively) but increased in the nasal cavities, tracheae, and lungs of *B. pertussis*-immune mice ($+10^{1.7}$, $+10^{1.5}$, $+10^{0.8}$ CFU, respectively) (Fig. 2D to F). Similar results were obtained when animals were rechallenged on day 70 postinoculation (data not shown), after the primary infection had been completely cleared from the lower respiratory tract. Furthermore, and consistent with previous reports, more efficient, but still incomplete, cross-protection was observed when bacterial numbers were analyzed 14 days postchallenge (data not shown). That being said, *B. parapertussis* was able to colonize hosts that had previously been infected with, and generated an effective immune response against, *B. pertussis*.

T-cell, but not antibody, responses are cross-reactive. The ability of *B. parapertussis* to infect *B. pertussis*-immune hosts suggested that the immune response was not effective against *B. parapertussis*, leading us to examine the cross-reactivity of T-cell and antibody responses. Mice were left uninoculated or inoculated with *B. pertussis* or *B. parapertussis*, and their spleens were excised 28 days postinoculation. The response of splenocytes to heat-killed *B. pertussis* or *B. parapertussis* was assessed by measuring the amount of IFN-γ produced, since this cytokine has been implicated in protective immunity to both bacteria (31, 32; D. N. Wolfe and E. T. Harvill, unpublished data). Splenocytes from naive and *B. parapertussis*-infected animals produced small amounts of IFN-γ in response to either species (Fig. 3), although the small amounts of IFN-γ produced by splenocytes from *B. parapertussis*-infected animals appeared to be protective (Fig. 1 and 2). Splenocytes from *B. pertussis*-infected mice responded similarly to both species by producing large amounts of IFN-γ (Fig. 3). Similar levels of IL-10 were produced in response to *B. pertussis* and *B. parapertussis*, regardless of the primary infection (data not shown). These data indicate that T cells induced by a *B. pertussis* infection are able to respond to *B. parapertussis* antigens. Furthermore, IFN-γ can contribute to the recruitment of neutrophils (52), which are important to the clearance of both pathogens (24, 62). Therefore, we quantified the numbers of neutrophils in the lungs of mice after a secondary infection with either species. Approximately 10^5 neutrophils were observed in the lungs of *B. pertussis*-convalescent mice upon *B. parapertussis* challenge compared to only 1×10^4 to 2×10^4 neutrophils in the lungs of naive or *B. parapertussis*-convalescent mice (data not shown), suggesting that these cells were being recruited to the lungs but were not effectively eliminating *B. parapertussis*.

To determine the ability of antibodies induced by one species to recognize the other, sera from *B. pertussis*- and *B. parapertussis*-convalescent mice were analyzed by ELISA and Western blotting. *B. pertussis* was recognized by sera raised against either species, although titers were approximately five times higher in *B. pertussis*-induced sera (titer ~ 1,000) than in *B. parapertussis*-induced sera (titer ~ 200) (Fig. 4A). *B. parapertussis* was recognized by *B. parapertussis*-induced antibodies (titer ~ 7,500), but titers in *B. pertussis*-induced sera were approximately 150-fold lower and

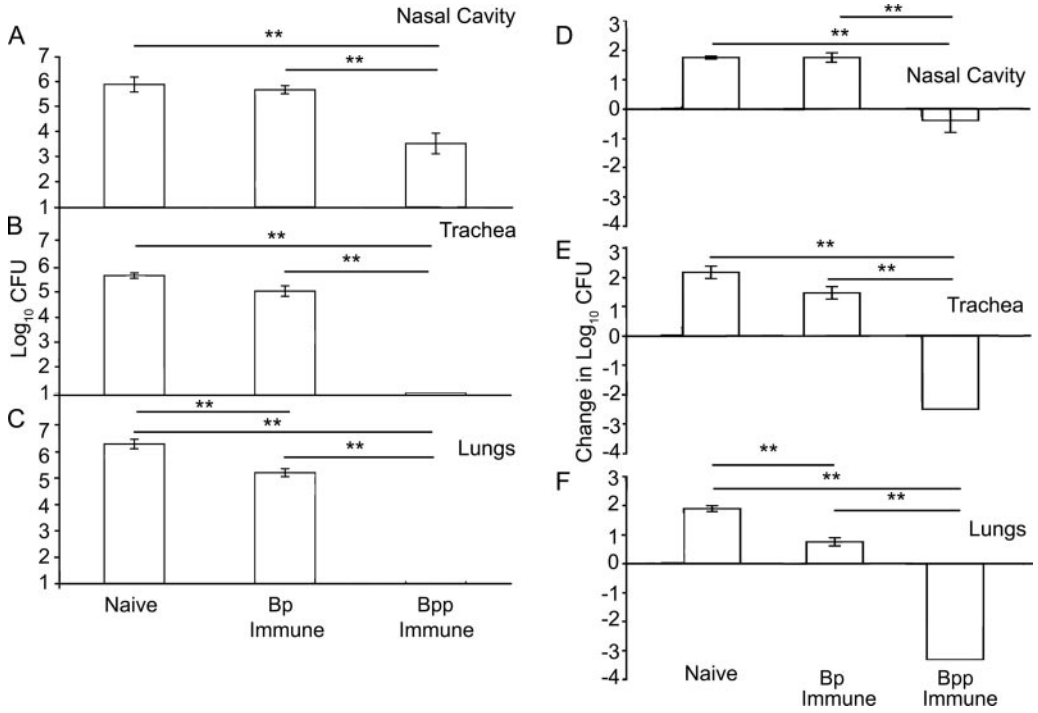

FIG. 2. *B. parapertussis* colonization of naive and immunized mice. C57BL/6 mice were inoculated with 5×10^5 CFU of *B. pertussis* (Bp) or *B. parapertussis* (Bpp). Immunized and naive mice were challenged 28 days later with 5×10^5 CFU of a gentamicin-resistant strain of *B. parapertussis* and sacrificed 3 days after secondary inoculation for the quantification of bacterial numbers in the nasal cavities (A), tracheae (B), and lungs (C). Values are expressed as $\log_{10}$ means ± standard deviations (SD). For day 3 colonization levels, the difference between each individual value and the mean $\log_{10}$ CFU approximately 10 min after inoculation in the nasal cavities (D), tracheae (E), and lungs (F) was calculated. These data are represented as the mean changes in $\log_{10}$ CFU ± SD. *, $P < 0.05$; **, $P < 0.01$.

near the lower limit of detection (titer ~ 50) (Fig. 4B), suggesting that the antibodies did not efficiently bind to *B. parapertussis*-coated plates. Western blots showed that antibodies induced by *B. pertussis* infection recognized antigens of both *B. pertussis* and *B. parapertussis* (Fig. 4C). The recognition of denatured, isolated *B. parapertussis* proteins in this assay is in contrast to the lack of recognition of whole bacteria in the ELISA (Fig. 4B), suggesting that shared protein antigens may not be exposed on the surface. *B. parapertussis*-induced antibodies also bound protein antigens of both species, as well as a large region of between 17 and 36 kDa present in *B. parapertussis* but not *B. pertussis*. This region was absent from an isogenic mutant of *B. parapertussis* lacking O antigen, and *B. pertussis* does not express an O antigen, indicating that this smear represents lipopolysaccharide containing O antigen (Fig. 4D). These data indicate that antibody responses to *B. pertussis* and *B. parapertussis* differ in the recognition of O antigen.

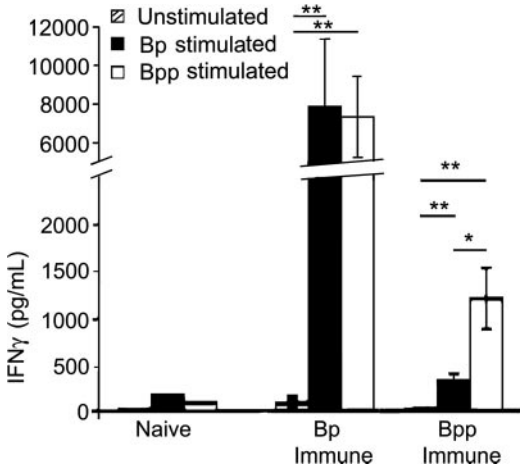

FIG. 3. IFN-γ production by splenocytes from naive or *B. pertussis*- or *B. parapertussis*-immune hosts. C57BL/6 mice were inoculated with 5×10^5 CFU of *B. pertussis* (Bp) or *B. parapertussis* (Bpp). Immunized and naive mice were sacrificed 28 days postinoculation, spleens were excised, and splenocytes were exposed to media alone (hatched bars), heat-killed *B. pertussis* (black bars), or heat-killed *B. parapertussis* (white bars) for 3 days. The supernatant was collected, and cytokine ELISAs were performed to quantify the levels of IFN-γ produced by splenocytes. Values are expressed as the means ± standard deviations. *, $P < 0.05$; **, $P < 0.01$.

Transfer of *B. parapertussis*-specific antibodies to *B. pertussis*-immune mice inhibits colonization by *B. parapertussis*. If *B. parapertussis* escapes cross-immunity by avoiding antibody recognition, adding *B. parapertussis* immune serum should confer protection to *B. pertussis*-immune mice. Mice were inoculated with *B. pertussis* and 28 days later were given a passive transfer of naive serum, *B. pertussis*-immune serum, or *B. parapertussis*-immune serum and challenged with *B. parapertussis*. Transferring naive or *B. pertussis*-induced serum had no measurable effect on *B. parapertussis* numbers, but transferring *B. parapertussis* immune serum to *B. pertussis*-immune hosts resulted in an approximately 100-fold reduction in bacterial numbers (Fig. 5). These data support the hypothesis that the lack of recognition by *B. pertussis*-induced antibodies allows *B. parapertussis* to avoid the immune response induced by its sister species.

O antigen inhibits the binding of *B. pertussis*-induced antibodies to *B. parapertussis*. We next sought to determine how *B.*

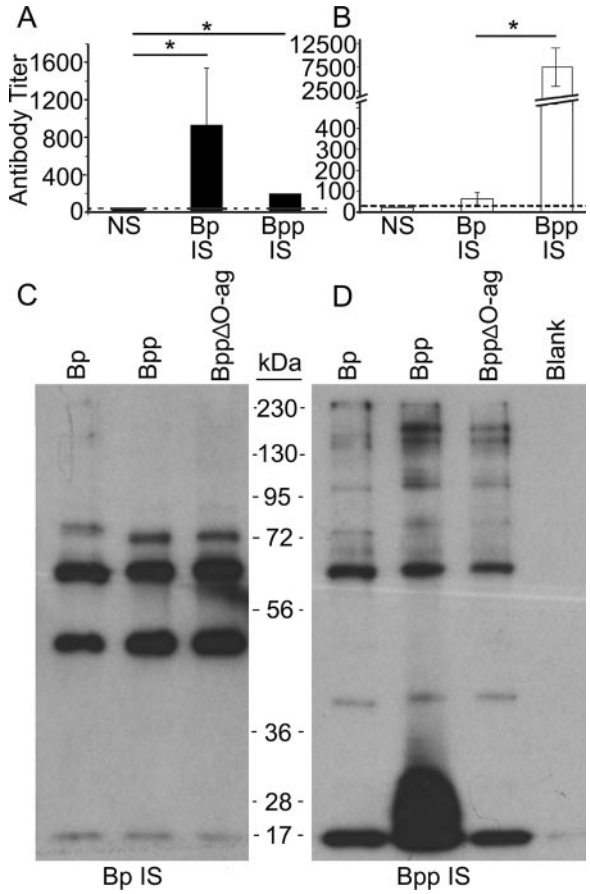

FIG. 4. Recognition of *B. pertussis* and *B. parapertussis* by antibodies from convalescent-phase serum. C57BL/6 mice were inoculated with 5×10^5 CFU of *B. pertussis* (Bp) or *B. parapertussis* (Bpp), and serum was collected from these mice 28 days later. ELISA was performed on serum from naive (NS), *B. pertussis*-infected (Bp IS), and *B. parapertussis*-infected (Bpp IS) mice to quantify titers of antibodies specific for *B. pertussis* (A) or *B. parapertussis* (B). The dashed line represents the lower limit of detection. Antibody titers from the ELISA are quantified as the $\log_{10}$ means of the end point titers ± standard deviations. *, $P < 0.05$; **, $P < 0.01$. Western blots of pooled *B. pertussis*-induced (C) and pooled *B. parapertussis*-induced (D) serum were used to determine if serum raised against one species recognized specific antigens of *B. pertussis*, *B. parapertussis*, or the *B. parapertussis* Δ*wbm* strain (BppΔO-ag).

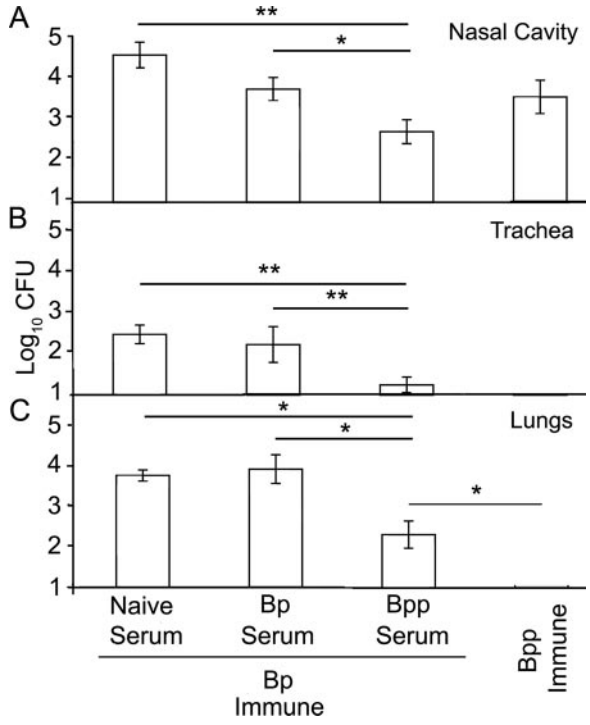

FIG. 5. Effect of passive transfer of immune serum to *B. pertussis*-immune mice on *B. parapertussis* numbers. C57BL/6 mice were inoculated with 5×10^5 CFU of *B. pertussis*. Twenty-eight days later, these mice were passively transferred sera from naive (naive serum), *B. pertussis*-infected (Bp serum), or *B. parapertussis*-infected (Bpp serum) mice and then challenged with *B. parapertussis*. *B. parapertussis*-convalescent mice (Bpp immune) were also challenged with *B. parapertussis* for comparison. CFU were quantified 3 days after secondary inoculation. Numbers of bacteria are expressed as $\log_{10}$ means ± standard deviations. *, $P < 0.05$; **, $P < 0.01$.

parapertussis avoids recognition by antibodies induced by such a closely related pathogen. *B. parapertussis*, but not *B. pertussis*, expresses O antigen (5), which has been shown to prevent the recognition of surface protein antigens by antibodies in other bacterial models (39, 53, 54, 57). We hypothesized that O antigen blocked the binding of *B. pertussis*-induced antibodies to common antigens. Live bacteria were used in ELISAs to determine if *B. pertussis*-induced antibodies bound more efficiently to intact *B. parapertussis* in the absence of O antigen. Sera from both *B. pertussis*- and *B. parapertussis*-infected mice recognized *B. pertussis* (titers ~ 1,000 and 600, respectively) (Fig. 6A). Titers of *B. pertussis*-induced antibodies that bound to live *B. parapertussis* were not significantly higher than the lower limit of detection, but titers of antibodies binding to live *B. parapertussis* lacking O antigen were approximately 15-fold higher (Fig. 6B). Thus, O antigen inhibits the binding of *B. pertussis*-induced antibodies to the surface of *B. parapertussis*.

O antigen enables *B. parapertussis* to colonize *B. pertussis*-immune hosts. If the blocking of antibody binding by O antigen allowed the evasion of *B. pertussis*-induced immunity, it would be expected that *B. pertussis* immunity would confer protection against O-antigen-deficient *B. parapertussis*. Mice were left uninfected or inoculated with *B. pertussis* or *B. parapertussis* and 28 days later challenged with wild-type or O-antigen-deficient *B. parapertussis*. Again, immunity to *B. pertussis* did not protect against wild-type *B. parapertussis* (Fig. 2 and 7). The O-antigen-deficient strain of *B. parapertussis* was present at approximately 10^6, 10^6, and 10^5 CFU in the nasal cavities, tracheae, and lungs of naive mice, respectively, but bacterial numbers were approximately 10-fold lower in the nasal cavities and 100,000-fold lower in the tracheae and lungs of *B. pertussis*-convalescent mice (Fig. 7). Thus, O antigen is required for *B. parapertussis* to evade *B. pertussis*-induced immunity and colonize *B. pertussis*-immune hosts.

DISCUSSION

B. parapertussis likely emerged into populations in which *B. pertussis* was endemic (4), making the ability to evade *B. pertussis*-induced immunity important to invading these popula-

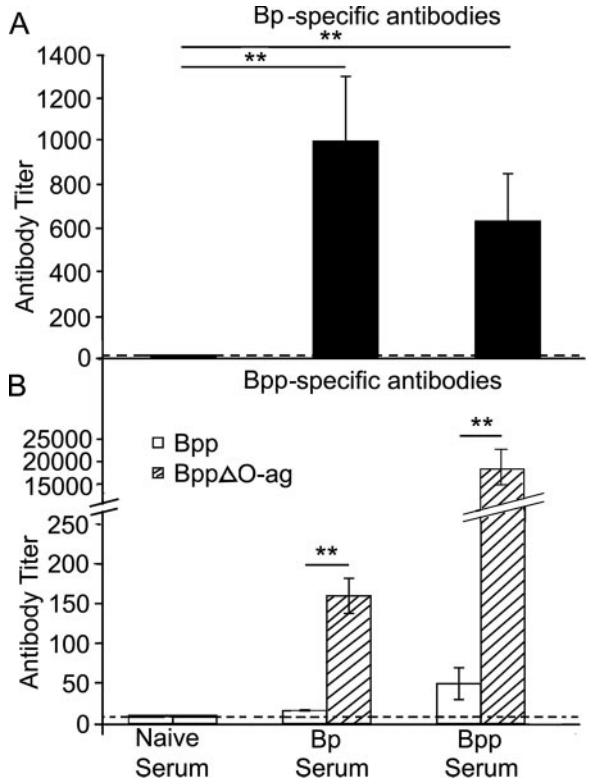

FIG. 6. Antibody recognition of live *B. pertussis* and *B. parapertussis* and O-antigen-deficient *B. parapertussis* by immune serum. C57BL/6 mice were inoculated with 5×10^5 CFU of *B. pertussis* (Bp) or *B. parapertussis* (Bpp), and serum was collected 28 days later. Sera from *B. pertussis* (Bp serum)- or *B. parapertussis*-infected mice (Bpp serum) or naive sera were examined by ELISA for their ability to bind to live *B. pertussis* (A) or *B. parapertussis* (B; white bars) or the O-antigen-deficient *B. parapertussis* (BppΔO-ag) (B; hatched bars). The dashed line represents the lower limit of detection. Antibody titers are expressed as the end point titers ± standard deviations. **, $P < 0.01$.

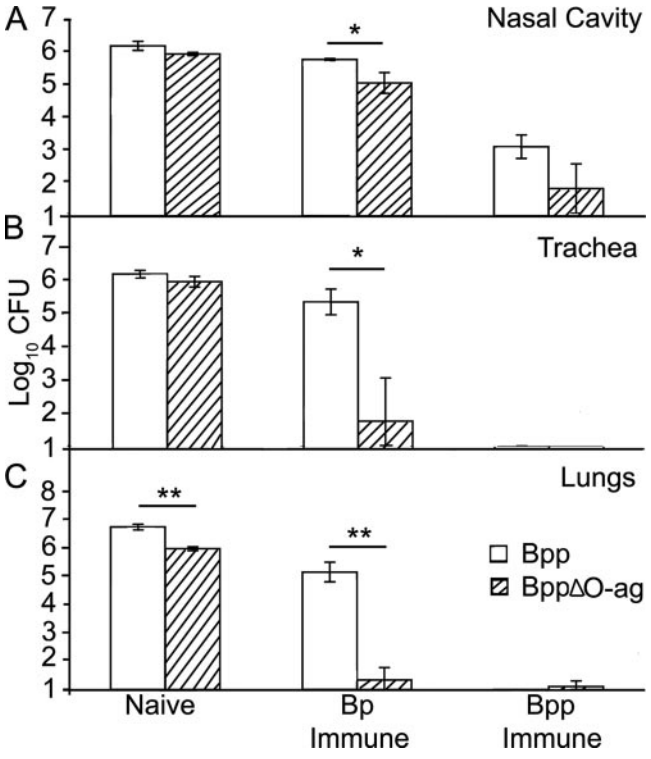

FIG. 7. Ability of the O-antigen-deficient strain of *B. parapertussis* to colonize *B. pertussis*-immune hosts. C57BL/6 mice were inoculated with 5×10^5 CFU of *B. pertussis* (Bp) or *B. parapertussis* (Bpp). Immunized and naive mice were challenged 28 days later with 5×10^5 CFU of *B. parapertussis* (white bars) or the O-antigen-deficient strain of *B. parapertussis* (BppΔO-ag; hatched bars). Mice were sacrificed 3 days after secondary inoculation for the quantification of bacterial numbers in the nasal cavities (A), tracheae (B), and lungs (C). All values are expressed as $\log_{10}$ means ± standard deviations. *, $P < 0.05$; **, $P < 0.01$.

tions. Here we have shown that *B. parapertussis* evades immunity to *B. pertussis* but that the evasion of cross-immunity is asymmetric. O antigen, which has been shown to mask surface antigens in other systems (39, 53, 54, 57), inhibits the binding of *B. pertussis*-induced antibodies and allows *B. parapertussis* to colonize *B. pertussis*-immune hosts. The observed asymmetric cross-immunity between *B. pertussis* and *B. parapertussis* is likely to contribute to their coexistence as endemic human pathogens.

Previous work has suggested efficient cross-immunity between these two species (58), but that study used a *B. pertussis* strain that, it has been suggested, is more similar to *B. bronchiseptica* and *B. parapertussis* than other *B. pertussis* strains (13, 55). More recent work has suggested that infection by an attenuated strain of *B. pertussis* may protect against subsequent *B. parapertussis* infections (38), but that study also observed that *B. parapertussis* numbers did not grow even in naive mice, whereas multiple studies have shown that *B. parapertussis* grows rapidly in naive mice (18, 25, 62). The attenuated strain used was also defective in producing pertussis toxin (38), a toxin that inhibits antibody production (6). Since low titers of *B. parapertussis*-specific antibodies in the serum of *B. pertussis* convalescent mice enabled the evasion of cross-immunity, pertussis toxin-mediated inhibition of antibody production may facilitate the evasion of immunity. It is important to point out that these two studies are in contrast to the voluminous research on *B. pertussis* vaccines, which show greatly diminished protection against *B. parapertussis* relative to protection against *B. pertussis* (11, 19, 29, 50, 61).

B. parapertussis O antigen limits the ability of antibodies from *B. pertussis*-immunized hosts to recognize surface antigens that are common between these two pathogens. An additional contributor to the asymmetrical cross-immunity could be that *B. pertussis* infection does not induce an antibody response to the appropriate protective antigen(s). *B. pertussis* does not express O antigen and thus does not induce O-antigen-specific antibodies. O antigen is a key protective antigen for a number of bacteria (23, 46), and antibodies against it may be important to protection against *B. parapertussis*. While O-antigen variation contributes to the coexistence of related bacterial species (43), we propose that O antigen is not essential to the infection of humans by *Bordetella*, as evidenced by its absence from *B. pertussis* strains, but the maintenance of O antigen by *B. parapertussis* may have allowed it to emerge in the niche of a *B. pertussis*-endemic population.

Host immunity has profound effects on the persistence of pathogens within host populations (15, 45, 47). Cross-reactive immunity between two species causes immune-mediated competition that may ultimately displace one from the host population. Despite the apparent asymmetrical cross-immunity between *B. pertussis* and *B. parapertussis*, these two species appear to circulate in out-of-phase cycles (26), and positive antibody titers to each have been observed in more than 50 percent of a population (33, 34). The question is, If there is asymmetrical cross-immunity between *B. pertussis* and *B. parapertussis*, why has the latter not displaced the former from human populations?

Whooping cough caused by *B. parapertussis* may be milder (3, 36); thus, assuming less coughing translates to less transmission, it is possible that *B. parapertussis* transmits less effectively than *B. pertussis*. This theory is supported by seroprevalence data indicating that approximately 60 percent of a population was seropositive for *B. parapertussis* but that over 90 percent of that population was seropositive for *B. pertussis* (33, 34). Furthermore, immunity induced by *B. pertussis* wanes after 5 to 10 years (60). The decay of immunity to *B. parapertussis* is not known, but cross-reacting immunity is likely to decay more rapidly than autologous immunity, potentially allowing subsequent infections by different strains or species (22).

The data presented here are evidence of a novel pattern of asymmetric cross-immunity conferred by *B. pertussis* and *B. parapertussis* that is consistent with the poor cross-protection conferred by vaccines (11, 19, 29, 50, 61) and that would affect the circulation of these pathogens throughout their host population. The evasion of *B. pertussis*-induced immunity by *B. parapertussis* may have allowed the latter to invade a population in which the former was endemic, leading to the observed coexistence. Importantly, it is possible that vaccination has changed, or is changing, the immune-mediated interactions between these two organisms. The effects of vaccines on *B. parapertussis* are unclear, but some have suggested that the prevalence of this species may be increasing despite, or even because of, vaccination efforts (29). In the face of these epidemiological and immunological complexities, it is important to establish the relative prevalence of *B. pertussis* and *B. parapertussis* as etiological agents in the ongoing resurgence of whooping cough.

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