

## Multiple Roles for *Bordetella* Lipopolysaccharide Molecules during Respiratory Tract Infection

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*Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* are closely related subspecies that cause respiratory tract infections in humans and other mammals and express many similar virulence factors. Their lipopolysaccharide (LPS) molecules differ, containing either a complex trisaccharide (*B. pertussis*), a trisaccharide plus an O-antigen-like repeat (*B. bronchiseptica*), or an altered trisaccharide plus an O-antigen-like repeat (*B. parapertussis*). Deletion of the *wlb* locus results in the loss of membrane-distal polysaccharide domains in the three subspecies of bordetellae, leaving LPS molecules consisting of lipid A and core oligosaccharide. We have used *wlb* deletion ( $\Delta wlb$ ) mutants to investigate the roles of distal LPS structures in respiratory tract infection by bordetellae. Each mutant was defective compared to its parent strain in colonization of the respiratory tracts of BALB/c mice, but the location in the respiratory tract and the time point at which defects were observed differed significantly. Although the  $\Delta wlb$  mutants were much more sensitive to complement-mediated killing *in vitro*, they displayed similar defects in respiratory tract colonization in C5<sup>-/-</sup> mice compared with wild-type (wt) mice, indicating that increased sensitivity to complement-mediated lysis is not sufficient to explain the *in vivo* defects. *B. pertussis* and *B. parapertussis*  $\Delta wlb$  mutants were also defective compared to wt strains in colonization of SCID-beige mice, indicating that the defects were not limited to interactions with adaptive immunity. Interestingly, the *B. bronchiseptica*  $\Delta wlb$  strain was defective, compared to the wt strain, in colonization of the respiratory tracts of BALB/c mice beginning 1 week postinoculation but did not differ from the wt strain in its ability to colonize the respiratory tracts of B-cell- and T-cell-deficient mice, suggesting that *wlb*-dependent LPS modifications in *B. bronchiseptica* modulate interactions with adaptive immunity. These data show that biosynthesis of a full-length LPS molecule by these three bordetellae is essential for the expression of full virulence for mice. In addition, the data indicate that the different distal structures modifying the LPS molecules on these three closely related subspecies serve different purposes in respiratory tract infection, highlighting the diversity of functions attributable to LPS of gram-negative bacteria.

Bordetellae are gram-negative bacteria that cause respiratory tract infections in mammals. *Bordetella pertussis* infects only humans, causing whooping cough (pertussis) in unvaccinated children and a milder coughing illness in adults. *B. parapertussis* also infects humans and causes a similar, albeit less severe, disease. *B. bronchiseptica* infects a variety of four-legged mammals, causing atrophic rhinitis in pigs, kennel cough in dogs, and snuffles in rabbits. Most *B. bronchiseptica* infections, however, are asymptomatic (8). Very small numbers of *B. bronchiseptica* organisms are sufficient to establish persistent infection in laboratory animals including rabbits, rats, and mice, allowing this subspecies to be used as a model for studies of naturally occurring host-pathogen interactions (1, 9, 14, 15). These three *Bordetella* subspecies are very closely related and express a similar set of virulence factors under the regulatory control of the BvgAS two-component system (24, 25). Virulence factors conserved between them, such as the putative adhesins filamentous hemagglutinin (FHA), pertactin, and fimbriae and the adenylate cyclase toxin, are likely to

perform functions required by all three subspecies for successful respiratory tract colonization (8).

Thus far, major phenotypic differences between bordetellae have not been shown to result from the presence or absence of pathogenicity islands, bacteriophage genomes, transposable elements, or plasmids. Instead, several Bvg-regulated loci found in the genomes of these three subspecies are differentially expressed. Examples include the genes and operons that encode a motility apparatus (2, 3), the pertussis toxin (7), and possibly a type III secretion system (28, 29). These differentially expressed factors are likely to contribute to subspecies-specific characteristics such as host specificity and the ability to cause pathology and to persist.

The lipopolysaccharide (LPS) molecules expressed on the surface of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* also differ substantially (4, 5, 17, 23). The observation that LPS structures are regulated by the BvgAS virulence control system suggests that these molecules play a role in respiratory tract infection (16, 17). *B. pertussis* LPS resolves as two bands when separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); these bands are designated bands A and B. Band B (Fig. 1) is composed of lipid A and a branched-chain core oligosaccharide. Addition of a trisaccharide to the band B form creates a larger LPS molecule (actually a lipooligosaccharide), referred to as band A (composed of band B plus

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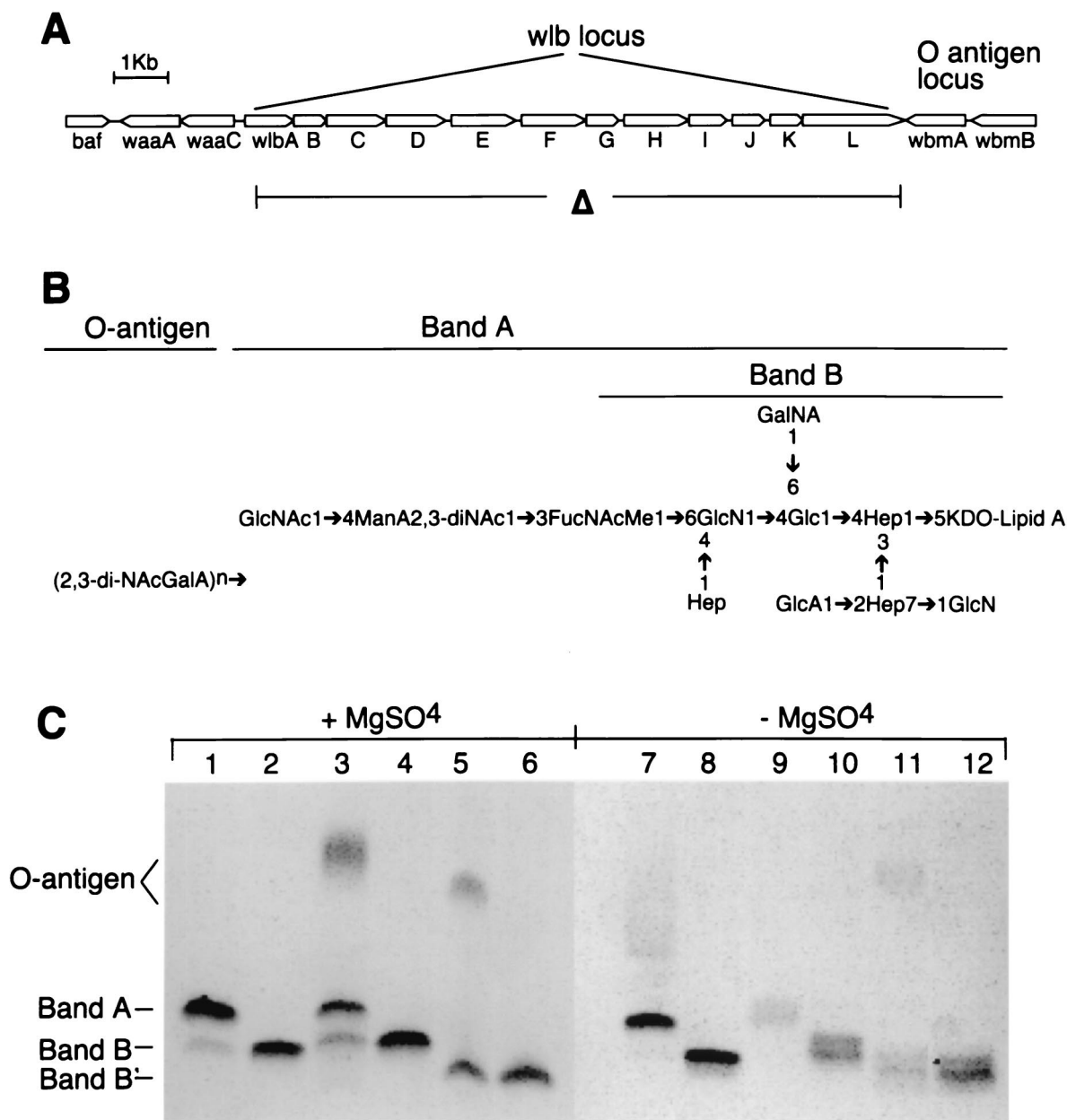


FIG. 1. (A) Diagram depicting the genetic organization of the *wlb* locus. The locus consists of 12 genes, *wlbA* to *wlbL*, that are required for expression of band A LPS (B). The locus is flanked on one side by Bvg accessory factor (*baf*) and *waaA* and *waaC*, which encode the LPS biosynthesis functions 2-keto-3-deoxyoctulosonic acid transferase and heptosyltransferase, respectively. In *B. bronchiseptica* and *B. paraperitussis*, the other side is flanked by the LPS O-antigen biosynthetic locus (23). In *B. pertussis*, this locus has been replaced by an insertion sequence. In the  $\Delta wlb$  mutants, the *wlb* locus has been deleted (as indicated in the diagram) and replaced by an antibiotic resistance cassette. (B) Schematic diagram of *Bordetella* LPS, as elucidated for *B. pertussis* strain 1414 (17). Band B, as viewed by SDS-PAGE (C), is composed of lipid A and a core containing several charged sugars including galactosaminuronic acid, glucuronic acid, and glucosamine. The *wlb* locus is required for the band B structure to be further substituted by a trisaccharide comprising *N*-acetylglucosamine (GlcNAc), 2,3-dideoxy-2,3-di-*N*-acetylmannosaminuronic acid (2,3-diNAcManA), and *N*-acetyl-*N*-methylfucosamine (FucNAcMe) to form band A, the predominant LPS form expressed by *B. pertussis*. *B. paraperitussis* expresses a smaller core, as suggested by the faster-migrating band B, and contains a mutated *wlbH*, which decreases the efficiency of GlcNAc transfer to the LPS, resulting in a truncated band A (14, 19). On wt *B. paraperitussis*, virtually all band A LPS is further substituted by O antigen and thus a distinct band A is not seen. Both *B. bronchiseptica* and *B. paraperitussis* synthesize a polymeric O antigen reported to consist of 2,3-dideoxy-2,3-di-*N*-acetylgalactosaminuronic acid (18). The attachment site for the O antigen is in the core but has not been precisely determined. 2,3-di-NAcGalA, 2,3-dideoxy-2,3-di-*N*-acetylgalactosaminuronic acid; GlcNAc, *N*-acetylglucosamine; ManA2,3-diNAc, 2,3-dideoxy-2,3-di-*N*-acetylmannosaminuronic acid; FucNAcMe, *N*-acetyl-*N*-methylfucosamine; GlcN, glucosamine; GalNA, galactosaminuronic acid; Glc, glucose; Hep, *L*-glycero-*D*-mannoheptose; GlcA, glucuronic acid; KDO, 2-keto-3-deoxyoctulosonic acid. (C) SDS-PAGE followed by silver staining of LPS prepared from wt *B. pertussis* (lanes 1 and 7), *B. pertussis*  $\Delta wlb$  (lanes 2 and 8), wt *B. bronchiseptica* (lanes 3 and 9), *B. bronchiseptica*  $\Delta wlb$  (lanes 4 and 10), wt *B. paraperitussis* (lanes 5 and 11), and *B. paraperitussis*  $\Delta wlb$  (lanes 6 and 12). Bacteria were grown in the presence or absence of MgSO<sub>4</sub>, as indicated, to produce Bvg<sup>-</sup> or Bvg<sup>+</sup> bacteria, respectively.

trisaccharide). *B. bronchiseptica* expresses LPS molecules that are very similar antigenically and electrophoretically to *B. pertussis* bands A and B, as well as a form containing an O-antigen-like homopolymer of 2,3-dideoxy-2,3-di-*N*-acetylgalactosaminuronic acid (2,3-di-NAcGalA), primarily in the Bvg<sup>-</sup> phase (12). *B. paraptussis* expresses a faster-migrating minimal molecule (band B'), as well as a large molecule containing the same O-antigen-like structure as *B. bronchiseptica*, and does not express the trisaccharide.

The *wlb* gene cluster, composed of 12 genes, is required for biosynthesis and addition of the trisaccharide in band A of *B. pertussis* and *B. bronchiseptica* and the O-antigen-like repeat in *B. bronchiseptica* and *B. paraptussis* (4, 6). Strains containing a *wlb* deletion mutation ( $\Delta wlb$ ) express only the smallest form of LPS that naturally occurs on these cells, band B on *B. pertussis* and *B. bronchiseptica* and band B' on *B. paraptussis*. The presence of these structurally diverse LPS molecules in otherwise closely related bacteria provides an opportunity to investigate the roles of these structures in infection. Here we have compared the wild-type (wt) and  $\Delta wlb$  strains of each subspecies in mouse respiratory tract infection models. All three mutants were defective in colonization of the respiratory tracts of BALB/c mice, but each was defective in different respiratory organs and/or at different periods during infection. Immunocompromised mice were used to investigate potential interactions between LPS structures and specific aspects of host immunity. Together, these results suggest that the distal structures of the LPS molecules of these three subspecies play different roles in infection. In *B. pertussis* they are required for efficient nasal colonization. In *B. paraptussis* they are required for initial colonization of the lungs. In *B. bronchiseptica* they are required for extended survival in the lower respiratory tracts of normal mice but not mice lacking adaptive immunity, suggesting that these structures are involved in resisting adaptive immune responses.

#### MATERIALS AND METHODS

**Bacteria.** Bacteria were maintained on Bordet-Gengou (BG) agar (Difco), inoculated into Stainer-Scholte broth at optical densities of 0.1 or lower, and grown to mid-log phase at 37°C on a roller drum for assays and inoculations. For Bvg<sup>-</sup> phase cultures, MgSO<sub>4</sub> was added to a final concentration of 40 mM. Wild-type (wt) strains (BP536, RB50, and CN2591) and the  $\Delta wlb$  derivatives of these three *Bordetella* subspecies have been described previously (4).

**LPS preparation and SDS-PAGE.** LPS was purified, using a modification of the method of Hitchcock and Brown (6, 16), from bacteria grown on BG agar plates with 15% defibrinated blood and supplemented with 200  $\mu$ g of streptomycin per ml. LPS was analyzed using the PAGE-Tricine buffer system by method of Lesse et al. (18). Silver staining was performed by the method of Tsai and Frasch (22).

**Animal experiments.** Female BALB/c mice, 4 to 6 weeks old, were obtained from Charles River Laboratories. SCID-beige mice were obtained from University of California Los Angeles facilities. C5<sup>-/-</sup> mice (B10.D2-H2<sup>d</sup>H2-T18<sup>Hc</sup>/nSnJ) and congenic controls (B10.D2-H2<sup>d</sup>H2-T18<sup>Hc</sup>/nSnJ) were obtained from Jackson Laboratory. Mice lightly sedated with halothane were inoculated with the indicated dose of bacteria by pipetting either 5 or 50  $\mu$ l of the inoculum onto the tip of the external nares. For time course experiments, groups of four animals were sacrificed on day 0, 3, 5, 7, 14, 21, 28, or 50 postinoculation. Colonization of various organs was quantified by homogenizing each tissue in phosphate-buffered saline (PBS), plating aliquots onto BG-blood agar, and counting colonies after 2 (*B. bronchiseptica*), 3 (*B. paraptussis*) or 4 (*B. pertussis*) days of incubation at 37°C. For the production of survival curves, after the progression of disease became clear, moribund animals were euthanized to prevent unnecessary suffering. Animals were handled in accordance with institutional guidelines. Statistical significance was determined using an unpaired *t* test.

**Serum-killing assays.** *Bordetella*-free rabbits were obtained from Charles River Laboratories, and their serum was confirmed to be free of *Bordetella*-specific antibodies by enzyme-linked immunosorbent assay and a Western immunoblot assay (data not shown). Immune serum was obtained from rabbits inoculated with *B. bronchiseptica* and colonized for 6 months (14). The serum was maintained complement active and was frozen in single-use aliquots at -80°C. Bacteria were grown in Stainer-Scholte broth to mid-log phase and

diluted in PBS to 100 CFU/ $\mu$ l. Serum was thawed on ice, and 90  $\mu$ l of serum, heat-killed serum, or PBS was mixed with 10  $\mu$ l of PBS containing 1,000 CFU of bacteria. The mixture was incubated at 37°C for 1 h. Samples were spread on BG agar plates and incubated for 2 to 4 days to determine bacterial numbers.

#### RESULTS

**Deletion of the *wlb* locus results in truncated LPS structures.** To examine the effect of deleting the *wlb* locus on LPS biosynthesis under Bvg<sup>+</sup> and Bvg<sup>-</sup> conditions, we compared, by silver-stained Tricine-SDS-PAGE, LPS structures of wt and  $\Delta wlb$  strains grown at 37°C on BG agar (Bvg<sup>+</sup> conditions) or BG agar with 40 mM MgSO<sub>4</sub> (Bvg<sup>-</sup> conditions). In agreement with previous reports, deletion of the *wlb* locus in *B. pertussis* resulted in the loss of the slower-migrating band A and the accumulation of the faster-migrating band B, representing the loss of the terminal trisaccharide (Fig. 1C, lanes 1, 2, 7, and 8) (6). wt *B. bronchiseptica* expressed molecules that comigrate with bands A and B, as well as slower-migrating molecules that contain O-antigen-like repeats (20), which are observed primarily in the Bvg<sup>-</sup> phase (lanes 3 and 9). Deletion of the *wlb* locus in *B. bronchiseptica* resulted in the loss of the slower-migrating forms of LPS and the accumulation of band B (lanes 4 and 10). *B. paraptussis* lacks band A but expresses a molecule that migrates somewhat faster than band B (band B'), as well as a larger form containing O-antigen-like repeats (lanes 5 and 11) (20). We have previously shown that deletion of genes required for O-antigen assembly in *B. paraptussis* results in accumulation of a species intermediate in size between bands A and B, probably representing the addition of a disaccharide by the *wlb* genes (20). This molecule is presumably efficiently substituted by O-antigen-like repeats in wt bacteria. Deletion of the entire *wlb* locus in *B. paraptussis* resulted in slight accumulation of the faster-migrating band B' and loss of the high-molecular-weight O-antigen-containing species (lanes 6 and 12). Under Bvg<sup>+</sup> growth conditions, additional bands appeared above bands B and B' in *B. bronchiseptica* and *B. paraptussis* wt and  $\Delta wlb$  strains, indicating that BvgAS-dependent modifications of these molecules occur that are not dependent on the presence of the terminal trisaccharide or the O-antigen-like repeat. Together, these data show that in all three subspecies, deletion of the *wlb* locus resulted in the loss of larger forms of LPS and an apparent accumulation of the smaller forms, bands B and B'. Because it is possible that changing the LPS structure could affect other molecules that associate with or pass through the outer membrane, we extensively characterized the  $\Delta wlb$  mutants in vitro. Deletion of the *wlb* locus did not affect growth rate, colony morphology, hemolysis, or expression of antigenic surface or secreted proteins as assessed by immunoblotting using sera from infected animals and serum raised against specific factors including pertactin, FHA, and adenylate cyclase toxin (data not shown). The  $\Delta wlb$  mutation did not affect FHA-dependent binding to L2 cells in vitro, indicating that FHA is present and functional on the surface of these cells (10).

***B. bronchiseptica* requires *wlb*-dependent LPS modification for efficient colonization of the trachea and lungs of BALB/c mice.** To determine the role of the trisaccharide and O-antigen-like structures of LPS in *B. bronchiseptica* respiratory tract colonization, BALB/c mice were inoculated intranasally with 5  $\times$  10<sup>5</sup> CFU of wt or  $\Delta wlb$  *B. bronchiseptica* in 50  $\mu$ l of PBS (Fig. 2). This inoculation regimen consistently delivers approximately 10<sup>5</sup> CFU to the nasal cavity, 10<sup>5</sup> CFU to the lungs, and 10<sup>3</sup> CFU to the trachea (Fig. 2 to 4, day 0). On subsequent days, *B. bronchiseptica* was recovered from all three sites at numbers larger than the initial inoculum, indicating that it was able to colonize and multiply throughout the respiratory tract

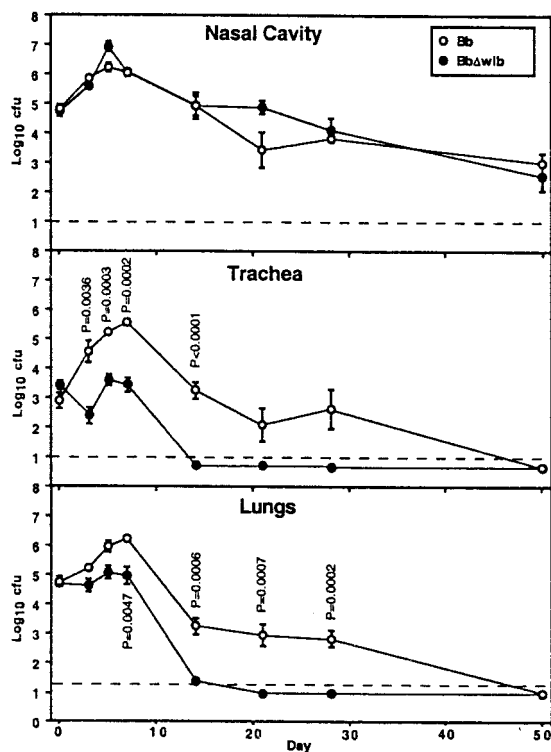


FIG. 2. The *B. bronchiseptica*  $\Delta wlb$  mutant is defective in tracheal and lung colonization in BALB/c mice. Groups of 4-week-old female mice were inoculated with  $5 \times 10^5$  CFU of either wt *B. bronchiseptica* or its  $\Delta wlb$  derivative delivered in a 50- $\mu$ l volume of PBS into the nares. Data points are presented as mean  $\log_{10}$  CFU and standard error. *P* values are shown where  $P < 0.02$ .

as we have previously shown (14). After 1 week the numbers of wt bacteria colonizing the respiratory tract decreased but bacteria were still recovered from both the trachea and the lungs 28 days after inoculation. In contrast, the  $\Delta wlb$  mutant did not increase in numbers in the trachea and lungs during the first week and could no longer be recovered from the lower respiratory tract by day 14 postinoculation. At multiple consecutive time points beginning on day 3, the numbers of  $\Delta wlb$  bacteria were significantly smaller ( $P < 0.01$ ) than those of the wt strain in both the trachea and the lungs. Although defective in tracheal and lung infection, the  $\Delta wlb$  strain was indistinguishable from the wt strain in colonization of the nasal cavity.

***B. paraptentussis* requires *wlb*-dependent LPS modification for efficient colonization of the trachea and lungs of BALB/c mice.** Clinical isolates of *B. paraptentussis* from humans are essentially clonal and appear to have diverged from *B. bronchiseptica* relatively recently (27). wt *B. paraptentussis* behaved similarly in most respects to wt *B. bronchiseptica* in BALB/c mice inoculated intranasally as above (compare Fig. 2 and 3). By day 3 postinoculation, wt *B. paraptentussis* was recovered from the nasal cavity, trachea, and lungs at numbers larger than the initial inoculum, indicating that it was able to colonize and multiply throughout the respiratory tract. After about 5 days, the numbers of wt bacteria began to decrease in all three organs and were below detectable levels in the trachea and lungs by day 21 and in the nasal cavity by day 50 postinoculation. The *B. paraptentussis*  $\Delta wlb$  strain was similar to the *B. bronchiseptica*  $\Delta wlb$  strain in that it was recovered at relatively constant numbers in the trachea for the first week and was absent by the second week. However, the *B. paraptentussis*  $\Delta wlb$  mutant differed from the *B. bronchiseptica* mutant in the mag-

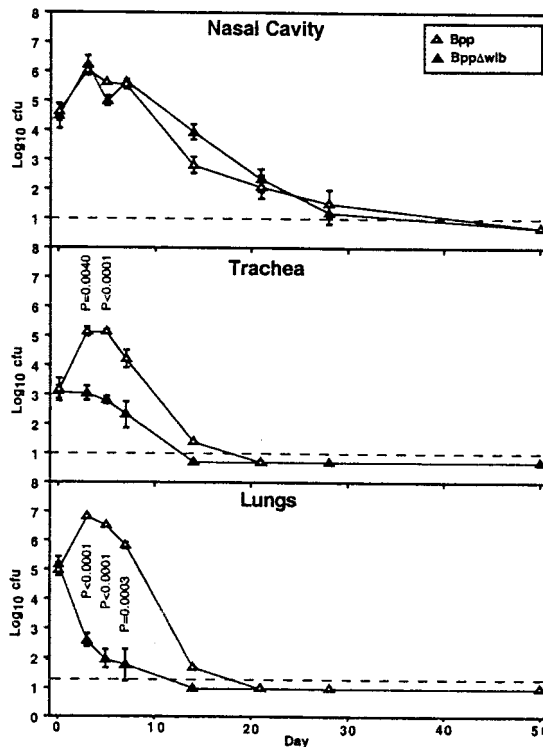


FIG. 3. The *B. paraptentussis*  $\Delta wlb$  mutant is defective in tracheal and lung colonization in BALB/c mice. Groups of 4-week-old female mice were inoculated with  $5 \times 10^5$  CFU of either wt *B. paraptentussis* or its  $\Delta wlb$  derivative delivered in a 50- $\mu$ l volume of PBS into the nares. Data points are presented as mean  $\log_{10}$  CFU and standard error. *P* values are shown where  $P < 0.02$ .

nitude of its defect in the lungs observed by day 3 postinoculation. On postinoculation days 3, 5, and 7, the numbers of *B. paraptentussis*  $\Delta wlb$  bacteria were 1/100 those of the wt strain in the trachea and 1/10,000 those of the wt strain in the lungs. The *B. paraptentussis*  $\Delta wlb$  mutant was similar to the *B. bronchiseptica*  $\Delta wlb$  mutant in that it was indistinguishable from the wt strain in colonization of the nasal cavity.

***B. pertussis* requires *wlb*-dependent LPS modification for efficient colonization of the nose, trachea, and lungs of BALB/c mice.** In the mouse model, *B. pertussis* efficiently colonizes the lungs but is defective, compared to *B. bronchiseptica*, in persistence in the nasal cavity (14). Although the wt *B. pertussis* strain was recovered from the nasal cavity, trachea, and lungs at numbers larger than the initial inoculum (Fig. 4, compare days 0 and 3), after a week the numbers of wt bacteria decreased and were below detectable levels in the trachea and nose by day 28 postinoculation. On day 28 postinoculation a small number of wt bacteria were recovered from the lungs, but by day 50 none were detected in any of the sites surveyed. The *B. pertussis*  $\Delta wlb$  mutant was similar to the *B. bronchiseptica* and *B. paraptentussis*  $\Delta wlb$  mutants in that it was recovered at relatively constant numbers in the trachea for the first week. In contrast to the *B. paraptentussis* mutant, the *B. pertussis*  $\Delta wlb$  mutant multiplied roughly 10-fold in numbers in the lungs during the first few days postinoculation and stayed at that approximate level for 2 weeks before declining and disappearing by day 28. Although neither the *B. bronchiseptica* nor the *B. paraptentussis*  $\Delta wlb$  mutants were defective in the nasal cavity of BALB/c mice, the *B. pertussis*  $\Delta wlb$  mutant was defective, with bacterial numbers approximately 1/1,000 those of the wt strain

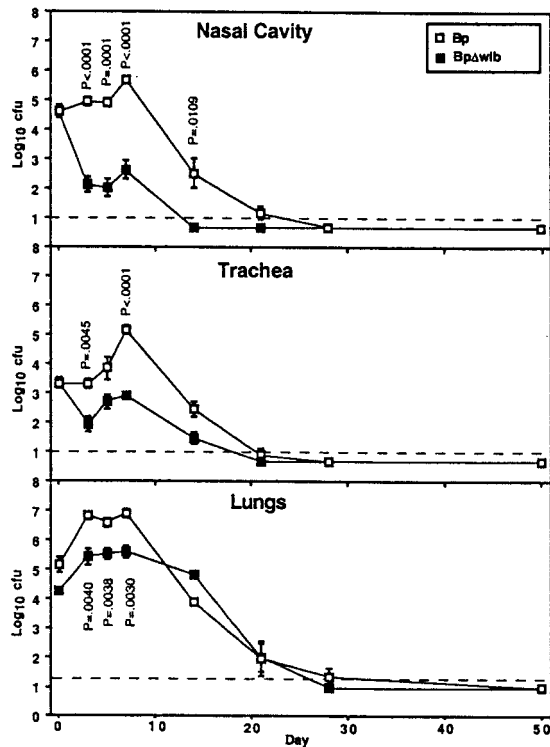


FIG. 4. The *B. pertussis*  $\Delta wlb$  mutant is defective in nasal, tracheal, and lung colonization in BALB/c mice. Groups of 4-week-old female mice were inoculated with  $5 \times 10^5$  CFU of either wt *B. pertussis* or its  $\Delta wlb$  derivative delivered in a 50- $\mu$ l volume of PBS into the nares. Data points are presented as mean log<sub>10</sub> CFU and standard error. *P* values are shown where *P* < 0.02.

on days 3, 5, and 7 and below detectable levels by day 14 postinoculation. The numbers of *B. pertussis*  $\Delta wlb$  mutant bacteria were decreased, compared to the wt strain, in all three organs (*P* < 0.01 at multiple time points) but were most severely defective in colonization of the nasal cavity, a phenotype not observed with  $\Delta wlb$  mutants of the other two subspecies.

**wlb-dependent LPS modification is required for lethal infection of SCID-beige mice by *B. bronchiseptica* and *B. paraperussis*.** The decrease in colonization levels throughout the respiratory tracts of BALB/c mice after day 7 postinoculation suggests that adaptive immunity is effective, albeit to different extents, against all three subspecies. We have previously used mice deficient in B and T cells (SCID, SCID-beige, and RAG-1 knockout mice) to identify interactions between bacterial virulence factors and innate versus adaptive immune responses (14, 15, 28). *B. bronchiseptica* is highly virulent in SCID, SCID-beige, and RAG-1 knockout mice, causing lethal systemic infections. *B. pertussis* is less virulent and does not kill these animals but persists for at least 200 days in the nose, trachea, and lungs (14). To identify potential roles for *wlb*-dependent LPS modifications in persistence and virulence, we compared the ability of these three *Bordetella* subspecies and their  $\Delta wlb$  mutants to infect SCID-beige mice (BALB/c genetic background), which are deficient in B and T cells as well as NK-cell activities. The beige mutation alone does not affect *B. bronchiseptica* infection, but in the context of SCID mice it speeds the progression of disease (15).

A low-dose, low-volume inoculum of 500 CFU in 5  $\mu$ l of PBS was delivered to the external nares, and survival was monitored over time. All six groups of mice remained healthy for 30 days,

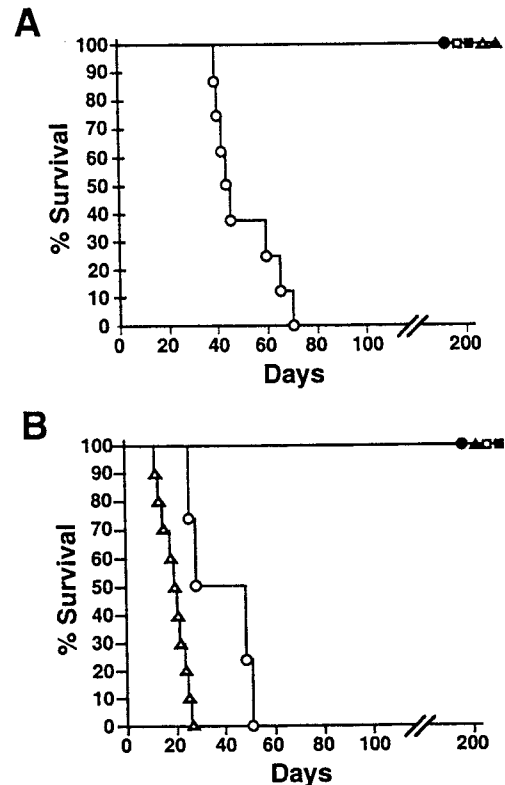


FIG. 5. Survival of SCID-beige mice following inoculation with *Bordetella* subspecies and their  $\Delta wlb$  mutants. SCID-beige mice were inoculated with either wt (open symbols) or  $\Delta wlb$  mutant (solid symbols) *B. bronchiseptica* (circles), *B. paraperussis* (triangles), or *B. pertussis* (squares). Percent survival is presented as a function of time following low-dose, low-volume intranasal inoculation with 500 CFU in a 5- $\mu$ l PBS droplet (A) or high-dose, high-volume intranasal inoculation with  $5 \times 10^5$  CFU in 50  $\mu$ l of PBS (B).

after which the animals infected with wt *B. bronchiseptica* began to display signs of illness such as piloerection, weight loss, hunched stature, listlessness, and, eventually, loss of responsiveness followed by death of 100% of the animals between days 40 and 70 postinoculation (Fig. 5A) (15). Mice inoculated with the *B. bronchiseptica*  $\Delta wlb$  mutant, in contrast, remained healthy for more than 200 days, indicating that *wlb*-dependent LPS modification is required for *B. bronchiseptica* to cause lethal infection in SCID-beige mice. Animals infected with either wt *B. pertussis*, *B. paraperussis*, or their  $\Delta wlb$  mutants remained healthy for more than 200 days.

SCID-beige mice were also inoculated with a high-dose high-volume regimen ( $5 \times 10^5$  CFU in 50  $\mu$ l of PBS), which seeds the entire respiratory tract with bacteria and deposits approximately  $10^5$  CFU in the lungs (Fig. 5B). *B. bronchiseptica* delivered by this regimen killed SCID-beige mice with slightly faster kinetics than the low-dose regimen did. wt *B. paraperussis*, which did not kill SCID-beige mice following low-dose inoculation, killed SCID-beige mice within 30 days following high-dose inoculation. *B. pertussis* and all three  $\Delta wlb$  mutants, however, did not cause lethal infections in these immunocompromised mice, even using the high-dose, high-volume regimen. These results highlight major differences in the virulence of *B. pertussis*, *B. paraperussis*, and *B. bronchiseptica* in this model and show that *wlb*-dependent LPS modification is required for the virulence of both *B. bronchiseptica* and *B. paraperussis* in SCID-beige mice.

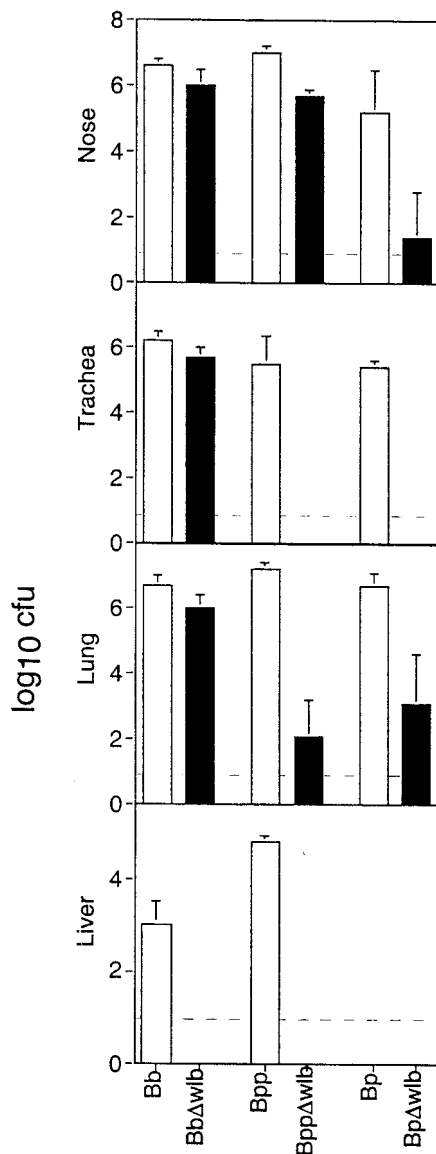


FIG. 6. Colonization of various tissues of SCID-beige mice by wt and  $\Delta wlb$  strains. Groups of four female 4-week-old SCID-beige mice were inoculated with  $5 \times 10^8$  CFU in  $50 \mu\text{l}$  of PBS. The mice were sacrificed and the colonization levels in various organs were determined on day 25 postinoculation for *B. pertussis* strains (Bp) and *B. bronchiseptica* strains (Bb) and day 21 for *B. parapertussis* strains (Bpp). Data are presented as mean  $\log_{10}$  CFU and standard error. The dashed line represents the lower limit of detection.

**Colonization levels in SCID-beige mice.** The three  $\Delta wlb$  mutants differed in the time points and anatomical locations at which they were defective in immunocompetent mice. To determine which, if any, of these colonization defects may relate to an ability to resist adaptive immune responses, we inoculated SCID-beige mice by the high-dose, high-volume regimen and determined colonization levels in various organs 25 days postinoculation. wt *B. bronchiseptica* was recovered at large numbers throughout the respiratory tract and at systemic sites including the livers of SCID-beige mice (Fig. 6) (14, 15). The *B. bronchiseptica*  $\Delta wlb$  strain was recovered at similar numbers to the wt strain throughout the respiratory tract but was not recovered from systemic sites such as the liver. Even at 100 and 200 days postinoculation, the *B. bronchiseptica*  $\Delta wlb$  strain

maintained a high level of colonization in the respiratory tract but was not recovered from the liver or spleen (data not shown). These data indicate that in SCID-beige mice, *wlb*-dependent LPS modification is not required for *B. bronchiseptica* colonization of the respiratory tract, suggesting that the defect of the  $\Delta wlb$  mutant in the lungs and trachea of BALB/c mice after 1 week is a result of B-cell, T-cell, and/or NK-cell function. The *B. bronchiseptica* *wlb* locus is, however, absolutely required for systemic spread to the liver and for lethal infection in SCID-beige mice.

SCID-beige mice inoculated with wt *B. parapertussis* contained large numbers of bacteria throughout the respiratory tract as well as the liver at 25 days postinoculation (Fig. 6). In BALB/c mice inoculated with the same dose of wt *B. parapertussis*, the trachea and lungs were cleared and the nasal cavity was nearly cleared by day 21 postinoculation (Fig. 3). These data indicate that immune mechanisms present in BALB/c mice but absent from SCID-beige mice are required to clear *B. parapertussis* infection. The  $\Delta wlb$  mutant was only slightly defective, compared to wt *B. parapertussis*, in colonization of the nasal cavities of SCID-beige mice but was severely defective in colonization of the tracheas and lungs of these animals. The striking defects of the *B. parapertussis*  $\Delta wlb$  mutant in the tracheas and lungs of SCID-beige mice and the similar defect observed within 3 days postinoculation in BALB/c mice indicate that *B. parapertussis* *wlb*-dependent LPS modification is required for colonization of these sites even in the absence of adaptive immunity. These data suggest that in *B. parapertussis* the *wlb*-dependent LPS modifications are involved in some aspect of infection other than interactions with adaptive immunity.

wt *B. pertussis* was recovered at large numbers throughout the respiratory tracts of SCID-beige mice 25 days postinoculation but not elsewhere in these animals (Fig. 6). We have previously shown that wt *B. pertussis* remains at relatively constant levels throughout the respiratory tracts of SCID-beige mice for at least 200 days but causes no overt signs of disease (14). The *B. pertussis*  $\Delta wlb$  mutant was defective, compared to the wt strain, in the noses, tracheas, and lungs of both BALB/c and SCID-beige mice, indicating that *wlb*-dependent LPS structures are required for some function other than evading the host adaptive immune responses.

***wlb*-dependent LPS modification is required by *B. bronchiseptica* and *B. parapertussis* for resistance to killing by naive serum.** wt *B. bronchiseptica* and *B. parapertussis*, but not their  $\Delta wlb$  mutants, were able to infect the livers of SCID-beige mice, suggesting that they could survive the antimicrobial activities of blood and lymph fluids encountered during transit to the liver. We reasoned that these  $\Delta wlb$  mutants could be defective in causing lethal systemic disease due to an increased susceptibility to antimicrobial factors in serum. We therefore compared wt and  $\Delta wlb$  strains of the three *Bordetella* subspecies for their survival in complement-active serum from *B. bronchiseptica*-free rabbits (naive serum). Bacteria ( $1,000$  CFU) from mid-log-phase liquid cultures, grown under Bvg<sup>+</sup> phase conditions, were incubated in  $100 \mu\text{l}$  of 90% serum to ensure that serum components were not limiting. wt *B. bronchiseptica* was not killed by a 1-h incubation at  $37^\circ\text{C}$  in 90% naive serum (Fig. 7). In contrast, the *B. bronchiseptica*  $\Delta wlb$  mutant was highly sensitive to naive serum (>99% of bacteria were killed), indicating that *wlb*-dependent LPS modification is required for *B. bronchiseptica* serum resistance. *B. parapertussis* was similar to *B. bronchiseptica* in that the wt strain was resistant but the  $\Delta wlb$  strain was killed (>99%) by naive serum. Unlike the other two subspecies, both wt and  $\Delta wlb$  *B. pertussis* strains were killed by naive serum in this assay.

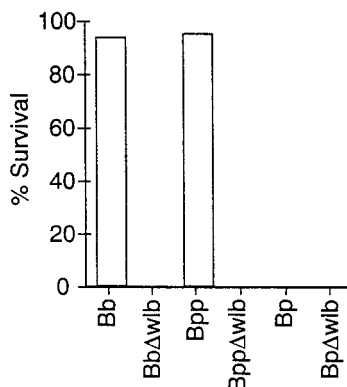


FIG. 7. Serum resistance of wt and  $\Delta wlb$  strains. Bacteria were grown to mid-log phase in Stainer-Scholte broth and diluted in PBS. A total of 1,000 bacteria were incubated at 37°C for 1 h in 100  $\mu$ l of 90% naive serum. Serum resistance is presented as the percent survival relative to a PBS control. Naive and immune sera are described in Materials and Methods.

Sera from *B. bronchiseptica*-infected rabbits (immune serum) were shown to contain antibodies recognizing all three *Bordetella* subspecies by ELISA and Western analysis (reference 14 and data not shown). wt *B. pertussis* and all three subspecies of  $\Delta wlb$  mutants, which were killed by naive serum, were killed by immune serum, as expected (data not shown). Immune serum also killed wt *B. bronchiseptica* (>98% killing) and *B. parapertussis* (>95% killing), which were not killed by naive serum, suggesting that antibody-mediated complement activation is responsible for the killing. Similar results were obtained in multiple experiments with naive sera from mice, rats, and rabbits or immune sera from mice, rats, rabbits, and humans. Inhibition of complement with EDTA or by heat treatment prevented killing in these assays (data not shown). Together, these data indicate that *wlb*-dependent LPS modification is required for resistance of *B. bronchiseptica* and *B. parapertussis* to the alternative complement cascade (naive serum) but is not sufficient for resistance to the classical pathway of complement (immune serum).

**Complement-deficient mice.** The sensitivity of the *B. bronchiseptica* and *B. parapertussis*  $\Delta wlb$  mutants to naive serum may be related to their defects in the BALB/c infection model. Serum components are present at low concentrations in normal respiratory fluids but increase in concentration in response to bacterial infection (13), suggesting that complement could affect colonization in the mouse model. SCID-beige mice contain normal complement levels, and the *B. parapertussis*  $\Delta wlb$  mutant was also defective in colonization of these animals, but the *B. bronchiseptica*  $\Delta wlb$  mutant colonized the respiratory tracts of SCID-beige animals to levels similar to those of the wt strain (Fig. 6). These data suggest that the colonization defect of the *B. bronchiseptica*  $\Delta wlb$  strain does not involve increased sensitivity to complement but that the defect of the *B. parapertussis*  $\Delta wlb$  mutant may do so. To directly determine if complement-mediated lysis contributes to control of respiratory tract infection by bordetellae, we compared the wt and  $\Delta wlb$  strains in congenic mouse strains with and without the ability to express the C5 component of complement (11, 19), which is required for the assembly of the membrane attack complex involved in complement-mediated lysis of gram-negative bacteria. In  $C5^{-/-}$  mice, complement activation by classical, alternative, or lectin pathways does not proceed past the point where these three pathways converge at the level of C5 activation prior to membrane attack complex formation, so

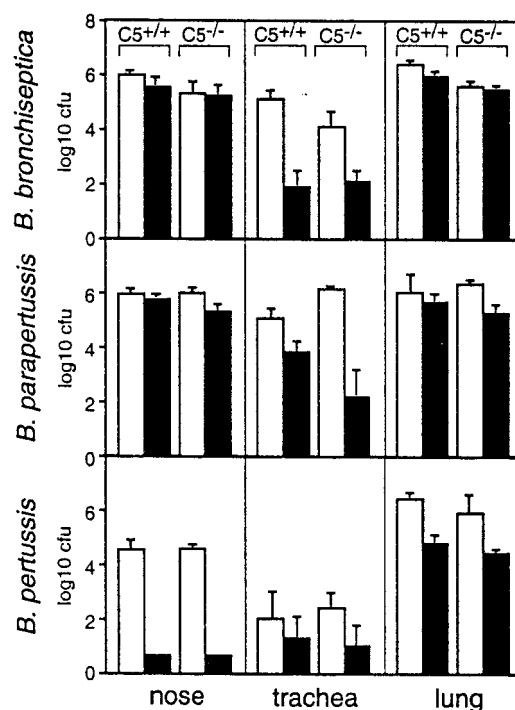


FIG. 8. *Bordetella*  $\Delta wlb$  mutants are defective in colonization of the respiratory tracts of complement-deficient mice. Groups of four 6-week-old female  $C5^{+/+}$  and  $C5^{-/-}$  mice were inoculated with  $5 \times 10^5$  CFU in 50  $\mu$ l of PBS. Colonization levels were determined in the nose, trachea, and lungs on day 5 postinoculation. Open bars represent wt strains, and solid bars represent  $\Delta wlb$  strains. Data are presented as mean log<sub>10</sub> CFU and standard error. The lower limit of detection is approximately 1.

that complement-mediated lysis is abrogated. In vitro, serum from  $C5^{+/+}$  mice killed *B. pertussis* and the  $\Delta wlb$  but not wt *B. bronchiseptica* and *B. parapertussis* strains but serum from congenic  $C5^{-/-}$  mice did not (data not shown). In vivo, the *B. bronchiseptica*  $\Delta wlb$  mutant was defective in colonization of the trachea, compared to the wt strain, in both  $C5^{+/+}$  and  $C5^{-/-}$  mice on day 5 postinoculation (Fig. 8). Apparently the defect in tracheal colonization is not due to the increased sensitivity of the  $\Delta wlb$  mutant to complement-mediated killing. Likewise, both *B. pertussis* and *B. parapertussis*  $\Delta wlb$  mutants showed as great a defect in colonization of  $C5^{-/-}$  mice as in  $C5^{+/+}$  mice, indicating that the defects are not due to increased sensitivity to complement-mediated killing. Interestingly, the *B. parapertussis*  $\Delta wlb$  strain, which was severely defective (1/10,000 of wt numbers) in the lungs of BALB/c mice on day 5 (Fig. 3) and SCID-beige mice (BALB/c genetic background) on day 21 (Fig. 6), showed little defect in the lungs of either  $C5^{+/+}$  or  $C5^{-/-}$  mice (B10.D2 genetic background). These data suggest that the substantial defect of the *B. parapertussis*  $\Delta wlb$  mutant in BALB/c lung colonization is not due to its sensitivity to complement-mediated killing but, rather, involves some other host factor that differs between mice of these two genetic backgrounds. The  $\Delta wlb$  mutants of all three subspecies, compared to their wt parent strains, were at least as defective in  $C5^{-/-}$  mice as in  $C5^{+/+}$  mice, indicating that the role of the *wlb* locus is not merely to render the bacteria complement resistant. Some other function of the *wlb*-dependent LPS modification is therefore involved in the observed phenotypes. The  $\Delta wlb$  strains did not show increased sensitivity to defensins (data not shown).

## DISCUSSION

LPS structures expressed by gram-negative bacteria have been proposed to contribute to infection by a variety of mechanisms including the mediation of adherence to host cells, antigenic variation, molecular mimicry, and induction of blocking antibodies (21). The LPS structures of *Bordetella* subspecies are less well studied and have not been documented to perform any of these functions, although they are modified in a Bvg-dependent manner, suggesting that they are involved in infection (26). We have compared *B. bronchiseptica*, *B. parapertussis*, and *B. pertussis* wt and  $\Delta wlb$  strains in vitro and in vivo, in normal and immunodeficient mice, to investigate the roles of the distal domains of their LPS molecules in infection.

All three  $\Delta wlb$  strains were similarly defective, compared to their wt parent strains, in colonization of the tracheas of BALB/c mice; they failed to increase in numbers in the trachea over the first week postinoculation and declined in numbers rapidly in the second week. Although deletion of the *wlb* locus increased the sensitivity of *B. bronchiseptica* and *B. parapertussis* to complement-mediated killing in vitro, the  $\Delta wlb$  mutants were defective in colonization of the tracheas of  $C5^{-/-}$  mice, in which complement-mediated killing is abrogated. These results indicate that complement-mediated lysis was not the only mechanism involved in the clearance of the  $\Delta wlb$  strains from the trachea, although it is possible that opsonization by C3, an event that does not require C5, could result in phagocytosis of the  $\Delta wlb$  strains. Interestingly, loss of distal LPS structures in the three *Bordetella* subspecies resulted in different phenotypes in the lungs and nasal cavities of various mice, supporting the view that these structures perform different functions for these three organisms.

Colonization of the lungs of BALB/c mice by the *B. bronchiseptica*  $\Delta wlb$  mutant was not significantly reduced compared to the wt strain until about 7 days postinoculation. The mutant was cleared from the lungs by 14 days postinoculation, whereas the wt strain was not cleared until day 50. Therefore, *wlb*-dependent LPS modification is not required for initial infection of the lungs by *B. bronchiseptica* but appears to contribute to persistence at a time consistent with the development of adaptive immune responses. In mice lacking adaptive immunity, the  $\Delta wlb$  mutant colonized the respiratory tract to similar levels to those of the wt strain, supporting the view that *wlb* gene products are primarily involved in resisting clearance by adaptive immune functions. Together, these data suggest that *wlb*-dependent LPS structures are required for *B. bronchiseptica* to resist clearance by the adaptive immune response. In preliminary experiments in  $\mu$ MT(C57BL/6-*Igh-6<sup>mt1Cgn</sup>*) mice, lacking B cells, both wt and  $\Delta wlb$  strains persist in the trachea and lungs, consistent with the primary role of *wlb*-dependent LPS modification being modulation of antibody-mediated bacterial clearance (data not shown). The inability of the  $\Delta wlb$  mutant to cause systemic infections and kill mice lacking adaptive immunity (SCID-beige mice) may be due to its increased sensitivity to serum complement-mediated killing compared to wt *B. bronchiseptica*. Although *B. bronchiseptica* is not believed to invade cells or tissues during natural infection, these observation may be relevant to aspects of infection that are not yet understood.

The role of distal LPS structures in *B. parapertussis* and *B. pertussis* infections appears to be substantially different from that in *B. bronchiseptica*. The *B. parapertussis*  $\Delta wlb$  mutant was recovered from the lungs of BALB/c mice at 1/10,000 the numbers of the wt strain by day 3 postinoculation. A defect of similar magnitude was observed for this mutant in the lungs of SCID-beige mice. These data indicate that, in contrast to *B.*

*bronchiseptica*, *wlb*-dependent LPS modification is required for *B. parapertussis* to colonize the lungs of mice even in the absence of adaptive immunity. Interestingly, the dramatic defect of the *B. parapertussis*  $\Delta wlb$  mutant in the lungs of BALB/c and SCID-beige mice (BALB/c genetic background) was not observed in  $C5^{+/+}$  or  $C5^{-/-}$  mice (B10.D2 genetic background). It is possible that *wlb*-dependent LPS modification protects bacteria from an antimicrobial activity present in BALB/c mice but absent in B10 mice. Alternatively, *wlb*-dependent LPS modification could be directly or indirectly required for binding to a receptor in BALB/c mice. B10 mice could express an alternative receptor(s), relieving the need for *wlb*-dependent LPS modification for lung infection in these animals.

Only *B. pertussis* required the *wlb* locus for efficient colonization of the nasal cavity. The *B. pertussis*  $\Delta wlb$  mutant was recovered from that organ at 1/1,000 the numbers of the wt strain. This defect was observed in the nasal cavities of BALB/c, SCID-beige,  $C5^{+/+}$ , and  $C5^{-/-}$  mice, indicating that the defect did not involve complement-mediated killing, adaptive immunity, or other interstrain differences between these animals. Interestingly, the *B. pertussis*  $\Delta wlb$  mutant was recovered from the tracheas and lungs of BALB/c mice at smaller numbers than the wt strain was on days 3, 5, and 7 postinoculation but thereafter the two strains were indistinguishable in these tissues. In contrast, the *B. bronchiseptica*  $\Delta wlb$  mutant was defective only on or after day 7 postinoculation and the *B. parapertussis*  $\Delta wlb$  mutant was severely defective at all time points from day 3 postinoculation onward.

The three *Bordetella* subspecies examined here are very closely related and are believed to have diverged from a common ancestor relatively recently (27). They maintain similar habitats, the respiratory tracts of mammals, and appear to differ primarily in their host ranges and in their ability to cause either acute disease with moderate to severe pathology or chronic infection with moderate to no pathology. Many of the factors involved in infection are highly conserved among the three subspecies, as would be expected for molecules that perform a function required for respiratory tract colonization in general. In contrast, the LPS structures of these three organisms appear to be substantially different. Although this could indicate that these structures have diverged because they are not important in infection, the data presented here show that they are critical to efficient respiratory tract colonization. The more likely explanation for these observations is therefore that these structures have evolved to meet the specific needs of these three organisms. For *B. bronchiseptica*, distal LPS structures are apparently required only when adaptive immunity has been generated, suggesting that they contribute to the persistent tracheal and lung infections characteristic of this subspecies. *B. pertussis* and *B. parapertussis*, which have independently shifted their host ranges to infect humans but do not cause persistent infections, appear to have modified their distal LPS structures to perform different roles. These functions are still required in SCID-beige mice, indicating that they are not limited to interactions with adaptive immunity. For *B. pertussis*, distal LPS structures are required for colonization of the nasal cavity of every mouse strain examined. For *B. parapertussis*, the  $\Delta wlb$  strain was severely defective (1/10,000 of wt levels) in colonization of the lungs of some mouse strains but not others. Together, these data suggest that the different distal structures modifying the LPS molecules on these three closely related subspecies serve different purposes in respiratory tract infection. Exchange of the genes involved in LPS assembly between the three subspecies will elucidate their roles in either common properties of respiratory tract colonization or subspecies-spe-



cific characteristics such as persistence, host specificity, and virulence.

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