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 (Δwlb) 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 Δwlb mutants were much more sensitive to complement-mediated killing in vitro, they displayed similar defects in respiratory tract colonization in C57−/− 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 Δwlb 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 Δwlb 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
FIG. 1. (A) Diagram depicting the genetic organization of the \textit{wlb} locus. The locus consists of 12 genes, \textit{wlbA} to \textit{wlbL}, that are required for expression of band A LPS (B). The locus is flanked on one side by Bvg accessory factor (\textit{baf}) and \textit{waaA} and \textit{waaC}, which encode the LPS biosynthesis functions 2-keto-3-deoxyoctulosonic acid transferase and heptosyltransferase, respectively. In \textit{B. bronchiseptica} and \textit{B. parapertussis}, the other side is flanked by the LPS O-antigen biosynthetic locus (23). In \textit{B. pertussis}, this locus has been replaced by an insertion sequence. In the \textit{Δwlb} mutants, the \textit{wlb} locus has been deleted (as indicated in the diagram) and replaced by an antibiotic resistance cassette. (B) Schematic diagram of \textit{Bordetella} LPS, as elucidated for \textit{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 \textit{wlb} locus is required for the band B structure to be further substituted by a trisaccharide comprising \textit{N}-acetylglucosamine (GlcNAc), 2,3-dideoxy-2,3-di-\textit{N}-acetylmannosaminuronic acid (2,3-diNacManA), and \textit{N}-acetyl-\textit{N}-methylfucosamine (FucNAcMe) to form band A, the predominant LPS form expressed by \textit{B. pertussis}. \textit{B. parapertussis} expresses a smaller core, as suggested by the faster-migrating band B, and contains a mutated \textit{wlbH}, which decreases the efficiency of GlcNAc transfer to the LPS, resulting in a truncated band A (14, 19). On wt \textit{B. parapertussis}, virtually all band A LPS is further substituted by O antigen and thus a distinct band A is not seen. Both \textit{B. bronchiseptica} and \textit{B. parapertussis} synthesize a polymeric O antigen reported to consist of 2,3-dideoxy-2,3-di-\textit{N}-acetylgalactosaminuronic acid (2,3-diNacGalA), and \textit{N}-acetyl-\textit{N}-methylfucosamine to form band A, the predominant LPS form expressed by \textit{B. pertussis}. \textit{B. parapertussis} expresses a smaller core, as suggested by the faster-migrating band B, and contains a mutated \textit{wlbH}, which decreases the efficiency of GlcNAc transfer to the LPS, resulting in a truncated band A (14, 19). On wt \textit{B. parapertussis}, virtually all band A LPS is further substituted by O antigen and thus a distinct band A is not seen. Both \textit{B. bronchiseptica} and \textit{B. parapertussis} synthesize a polymeric O antigen reported to consist of 2,3-diNacGalA, 2,3-dideoxy-2,3-di-\textit{N}-acetylgalactosaminuronic acid; GlcNAc, \textit{N}-acetylglucosamine; ManA2,3-diNacA, 2,3-dideoxy-2,3-di-\textit{N}-acetylgalactosaminuronic acid; FucNAcMe, \textit{N}-acetyl-\textit{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 \textit{B. pertussis} (lanes 1 and 7), \textit{B. pertussis} \textit{Δwlb} (lanes 2 and 8), wt \textit{B. bronchiseptica} (lanes 3 and 9), \textit{B. bronchiseptica} \textit{Δwlb} (lanes 4 and 10), wt \textit{B. parapertussis} (lanes 5 and 11), and \textit{B. parapertussis} \textit{Δwlb} (lanes 6 and 12). Bacteria were grown in the presence or absence of MgSO$_4$, as indicated, to produce Bvg$^+$ or Bvg$^-$ 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-N-acGalaA), primarily in the Bvg phase (12). *B. parapertussis* 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. parapertussis* (4, 6). Strains containing a *wlb* deletion mutation (∆*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. parapertussis*. 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 ∆*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 bands A and B 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. parapertussis* 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 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 *B. pertussis* and *B. bronchiseptica* the O-antigen-like repeat in *B. bronchiseptica* and *B. parapertussis* resulted in the loss of the slower-migrating band B, the representing the loss of the terminal trisaccharide (Fig. 1C). 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. parapertussis* 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. parapertussis* 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. parapertussis* 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* growth conditions, additional bands appeared above bands B and B' in *B. bronchiseptica* and *B. parapertussis* wt and ∆*wlb* strains, indicating that *Bvg*-AS-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 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 ∆*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 ∆*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).

**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* and *Bvg* conditions, we compared, by silver-stained Tricine-SDS-PAGE, LPS structures of wt and ∆*wlb* strains grown at 37°C on BG agar (∆*wlb* conditions) or BG agar with 40 mM MgSO₄ (*Bvg* 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* 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. parapertussis* 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. parapertussis* 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. parapertussis* 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* growth conditions, additional bands appeared above bands B and B' in *B. bronchiseptica* and *B. parapertussis* wt and ∆*wlb* strains, indicating that *Bvg*-AS-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 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 ∆*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 ∆*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 × 10⁵ CFU of wt or ∆*wlb* *B. bronchiseptica* in 50 μl of PBS (Fig. 2). This inoculation regimen consistently delivers approximately 10⁵ CFU to the nasal cavity, 10² CFU to the lungs, and 10³ 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.

Materials and Methods...
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 Δwlb 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 Δwlb 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 Δwlb strain was indistinguishable from the wt strain in colonization of the nasal cavity.

_B. parapertussis_ requires _wlb_-dependent LPS modification for efficient colonization of the trachea and lungs of BALB/c mice. Clinical isolates of _B. parapertussis_ from humans are essentially clonal and appear to have diverged from _B. bronchiseptica_ relatively recently (27). wt _B. parapertussis_ 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. parapertussis_ 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. parapertussis_ Δwlb strain was similar to the _B. bronchiseptica_ Δwlb 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. parapertussis_ Δwlb mutant differed from the _B. bronchiseptica_ mutant in the magnitude of its defect in the lungs observed by day 3 postinoculation. On postinoculation days 3, 5, and 7, the numbers of _B. parapertussis_ Δwlb 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. parapertussis_ Δwlb mutant was similar to the _B. bronchiseptica_ Δwlb 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_ Δwlb mutant was similar to the _B. bronchiseptica_ and _B. parapertussis_ Δwlb mutants in that it was recovered at relatively constant numbers in the trachea for the first week. In contrast to the _B. parapertussis_ mutant, the _B. pertussis_ Δwlb 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. parapertussis_ Δwlb mutants were defective in the nasal cavity of BALB/c mice, the _B. pertussis_ Δwlb mutant was defective, with bacterial numbers approximately 1/1,000 those of the wt strain.
on days 3, 5, and 7 and below detectable levels by day 14 postinoculation. The numbers of *B. pertussis* on days 3, 5, and 7 and below detectable levels by day 14 CFU and standard error.

**FIG. 4.** The *B. pertussis* Δ*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 × 10⁵ CFU of either wt *B. pertussis* or its Δ*wlb* derivative delivered in a 50-µl volume of PBS into the nares. Data points are presented as mean log₁₀ CFU and standard error. P values are shown where P < 0.02.

**FIG. 5.** Survival of SCID-beige mice following inoculation with *Bordetella* subspecies and their Δ*wlb* mutants. SCID-beige mice were inoculated with either wt (open symbols) or Δ*wlb* mutant (solid symbols) *B. bronchiseptica* (circles), *B. parapertussis* (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-µl PBS droplet (A) or high-dose, high-volume intranasal inoculation with 5 × 10⁵ CFU in 50 µl of PBS (B).

**A**

**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* Δ*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. parapertussis*, or their Δ*wlb* mutants remained healthy for more than 200 days.

SCID-beige mice were also inoculated with a high-dose high-volume regimen (5 × 10⁵ CFU in 50 µl of PBS), which seeds the entire respiratory tract with bacteria and deposits approximately 10⁵ 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. parapertussis*, which did not kill SCID-beige mice following low-dose inoculation, killed SCID-beige mice within 30 days after high-dose inoculation. *B. pertussis* and all three Δ*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. parapertussis*, and *B. bronchiseptica* in this model and show that *wlb*-dependent LPS modification is required for the virulence of both *B. bronchiseptica* and *B. parapertussis* in SCID-beige mice.
colonization defects may relate to the wt strain throughout the respiratory tract 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 Δwlb 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 modifications 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 Δwlb 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 Δwlb mutants could be defective in causing lethal systemic disease due to an increased susceptibility to antimicrobial factors in serum. We therefore compared wt and Δwlb 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− phase conditions, were incubated in 100 μl of 90% serum to ensure that serum components were not limiting. wt B. bronchiseptica was not killed by a 1-h incubation at 37°C in 90% naive serum (Fig. 7). In contrast, the B. bronchiseptica Δwlb 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 Δwlb strain was killed (>99%) by naive serum. Unlike the other two subspecies, both wt and Δwlb B. pertussis strains were killed by naive serum in this assay.
activation prior to membrane attack complex formation, so the point where these three pathways converge at the level of C5 is classical, alternative, or lectin pathways does not proceed past 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 that complement-mediated lysis is abrogated. In vitro, serum from C5−/− mice killed B. pertussis and the Δwlb mutant 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 Δwlb mutant was defective in colonization of the trachea, compared to the wt strain, in both C5−/− and C5−/− mice as in C5−/− mice. Apparently the defect in tracheal colonization is not due to the increased sensitivity of the Δwlb mutant to complement-mediated killing. Likewise, both B. pertussis and B. parapertussis Δwlb 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 Δwlb strain, which was severely defective (1/10,000 of wt numbers) in the lungs of BALB/c mice on day 5 postinoculation, showed little defect in the lungs of either C5−/− or C5−/− mice (B10.D2 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 Δwlb 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 Δwlb 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 Δwlb strains did not show increased sensitivity to defensins (data not shown).

FIG. 7. Serum resistance of wt and Δwlb 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 μl 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.

FIG. 8. Bordetella Δwlb 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 × 10⁸ CFU in 50 μl 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 Δwlb strains. Data are presented as mean log₁₀ CFU and standard error. The lower limit of detection is approximately 1.
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 *Δwlb* 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 *Δwlb* strains were similarly defective, compared to their wt parent strains, in colonization of the tracheae 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 *Δwlb* mutants were defective in colonization of the tracheae of C57BL/6 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 *Δwlb* 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 *Δwlb* 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* *Δwlb* 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 30. 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 *Δwlb* 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 μMT(C57BL/6-Igh-tg6tm1Cgn) mice, lacking B cells, both wt and *Δwlb* 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 *Δwlb* 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 observations 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* *Δwlb* 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* *Δwlb* mutant in the lungs of BALB/c and SCID-beige mice (BALB/c genetic background) was not observed in C57BL/6 or C57BL/6 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* *Δwlb* mutant was recovered from that organ at 1/100,000 the numbers of the wt strain. This defect was observed in the nasal cavities of BALB/c, SCID-beige, C57BL/6, and C57BL/6 mice, indicating that the defect did not involve complement-mediated killing, adaptive immunity, or other interstrain differences between these animals. Interestingly, the *B. pertussis* *Δwlb* mutant was recovered from the tracheae 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* *Δwlb* mutant was defective only on or after day 7 postinoculation and the *B. parapertussis* *Δwlb* 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 *Δwlb* 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-
sific characteristics such as persistence, host specificity, and virulence.

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