In Vitro and In Vivo Characterization of a *Bordetella bronchiseptica* Mutant Strain with a Deep Rough Lipopolysaccharide Structure

Federico Sisti,1 Julia Fernández,1,2 María Eugenia Rodríguez,2 Antonio Lagares,1 Nicole Guiso,3 and Daniela Flavia Hozbor1,2,∗

Instituto de Bioquímica y Biología Molecular1 and Centro de Investigación y Desarrollo en Fermentaciones Industriales,2 Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 1900 La Plata, República Argentina, and Unité des Bordetella, Institut Pasteur, 75724 Paris Cédex 15, France3

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*Bordetella bronchiseptica* is closely related to *Bordetella pertussis*, which produces respiratory disease primarily in mammals other than humans. However, its importance as a human pathogen is being increasingly recognized. Although a large amount of research on *Bordetella* has been generated regarding protein virulence factors, the participation of the surface lipopolysaccharide (LPS) during *B. bronchiseptica* infection is less understood. To get a better insight into this matter, we constructed and characterized the behavior of an LPS mutant with the deepest possible rough phenotype. We generated the defective mutant *B. bronchiseptica* LP39 on the *waaC* gene, which codes for a heptosyl transferase involved in the biosynthesis of the core region of the LPS molecule. Although in *B. bronchiseptica* LP39 the production of the principal virulence determinants adenylate cyclase-hemolysin, filamentous hemagglutinin, and pertactin persisted, the quantity of the two latter factors was diminished, with the levels of pertactin being the most greatly affected. Furthermore, the LPS of *B. bronchiseptica* LP39 did not react with sera obtained from mice that had been infected with the parental strain, indicating that this defective LPS is immunologically different from the wild-type LPS. In vivo experiments demonstrated that the ability to colonize the respiratory tract is reduced in the mutant, being effectively cleared from lungs within 5 days, whereas the parental strain survived at least for 30 days. In vitro experiments have demonstrated that, although *B. bronchiseptica* LP39 was impaired for adhesion to human epithelial cells, it is still able to survive within the host cells as efficiently as the parental strain. These results seem to indicate that the deep rough form of *B. bronchiseptica* LPS cannot represent a dominant phenotype at the first stage of colonization. Since isolates with deep rough LPS phenotype have already been obtained from human *B. bronchiseptica* chronic infections, the possibility that this phenotype arises as a consequence of selection pressure within the host at a late stage of the infection process is discussed.

Bacterial surface polysaccharides are important contributors to the processes of bacterium-host interaction, including symbiotic and pathogenic relationships (30). Among these polysaccharides, the lipopolysaccharide (LPS) is the major component of the outer membrane of gram-negative bacteria. The LPSs of the three most studied *Bordetella* species, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, share basic structural features in that they each have a lipid A domain and a branched-chain core oligosaccharide. However there are differences, since *B. parapertussis* and *B. bronchiseptica* synthesize an O-antigen structure consisting of a polymer of the single sugar residue 2,3-di-N-acetylglactosaminuronic acid, whereas *B. pertussis* does not (31).

*B. bronchiseptica* is currently acquiring relevance because of its increased importance as a human pathogen (20, 48). This microorganism colonizes the ciliated epithelium of the respiratory tract of the host and establishes chronic infections (20). It has been speculated that the development of such chronic infections may partially depend on the ability of bacteria to develop adaptive phenotypic changes in response to variable stimuli. This bacterial capacity could be related to two members of the two-component family of signal transduction protein, the *bv* (*Bordetella* virulence gene) (4, 13, 16, 34) and *ris* (regulator of intracellular stress response) loci (5, 9, 20, 26). In agreement with this hypothesis, it was reported that in a patient with bronchopneumonia in whom *B. bronchiseptica* persisted over a period of 2 years, bacteria isolated from the initial period of infection produced toxins and adhesins (14, 22, 27), while successive isolates produced only adhesins (20).

Concomitant with this variation, the lipopolysaccharide structure shifts from a smooth to a rough/deep rough phenotype (21). The same LPS phenotypic change along the course of the infection was observed in another human patient with persistent *B. bronchiseptica* infection (unpublished results). Bacterial isolates obtained from different hosts also present different lengths of LPS molecules (29), pointing out that the LPS variation in vivo might be common in long-term infections. In addition, the observation that LPS structure varies in vivo suggests that this molecule plays different roles during the different stages of *B. bronchiseptica* infection (6, 23).

In other gram-negative bacteria, it was demonstrated that the LPS structure strongly influences the synthesis and/or secretion of certain proteins (7, 10, 15, 40, 44). In the case of *Bordetella*, current evidence indicates that the expression of both components, LPS and protein virulence determinants, is modified by the environment (13, 32, 34, 42). At present, how-
ever, the linkage between the different structures of the LPS, in particular the deep rough phenotype, with the expression of virulence factors and its significance in the whole infection process remain to be established.

In order to gain an insight into the role of LPS in *B. bronchiseptica* infection, we constructed the deepest possible rough LPS phenotype by site-specific insertional mutagenesis on the wasC gene, which codes for the glycosyltransferase responsible for the addition of the first heptose residue to 3-deoxy-D-manno-octulosonic acid (15, 31, 36). The *B. bronchiseptica* wasC mutant obtained was characterized within the framework of virulence determinant production and its in vitro and in vivo behavior.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *Escherichia coli* strains DH5α (Bethesda Res. Lab) and S17-1 (37) were cultured in Luria-Bertani (LB) medium supplemented, when appropriate, with ampicillin or kanamycin at a final concentration of 100 or 25 μg ml⁻¹, respectively. *B. bronchiseptica* strain 9.73 (Collection de l’Institut Pasteur designation) was grown on Bordet Gengou agar (Difco) supplemented with 15% (vol/vol) defibrinated fresh sheep blood (BGA medium) at 36°C for 48 h. Then it was replaced in the same medium for 24 h. For mutant selection, BGA was supplemented with streptomycin (200 μg ml⁻¹) and kanamycin (75 μg ml⁻¹). For LPS extraction, subcultures were grown in Stainer-Scholte (SS) liquid medium (39) for 20 h at 36°C until the optical density measured at 650 nm reached 1.0.

In order to label bacteria to be able to study bacterial infection of mice, we introduced plasmid pGBK514 (45), which codes for kanamycin resistance and for the green fluorescent protein, into the wild-type *B. bronchiseptica* strain by conjugation. This plasmid was kindly provided by Alison Weiss (Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati). For immunoblot analysis, bacteria grown in BGA were resuspended in saline at a concentration of 2 × 10⁹ CFU ml⁻¹ diluted in Lactem buffer (38), and boiled for 15 min.

**PCR and recombinant DNA techniques.** Based on previously reported consensuses sequences (2, 3, 7, 16, 36) (*B. bronchiseptica* cosmid Bl/LPS AI007747), we designed two single-stranded oligonucleotide primers, wasC_G (5'-TTCACT ACGCC CCTG-3', where I is inosine) and wasC_R (5'-CCAGATGACAGGTGTT-3'). These two oligonucleotides were provided by DNAgency, Inc. (Malvern, N.Y.). We then amplified a 200-bp fragment corresponding to an internal sequence of the wasC gene. This PCR product was cloned into the shuttle plasmid pGem T-Easy (Ags, IacZ, Promega). After ligation, a recombinant fragment from this plasmid was released with EcoRI and cloned into the EcoRI site of the Bordetella sidue plasmid pKI8800 (Km) (35).

We performed conjugation incubations in BGA plus 10 mM MgCl₂ using *B. bronchiseptica* 9.73 ΔSar as the recipient and *E. coli* S17-1 containing the recombinant suicide plasmid as the donor, and thereby colonies were selected for single gene crossovers on kanamycin plus streptomycin. We then analyzed the detergent sensitivity of the resultant colonies of transconjugants on Stainer-Scholte solid medium supplemented with 0.02% (wt/vol) SDS. From an SDS-resistant colony, recombinant plasmids, hereafter referred to as *B. bronchiseptica* wasC mutants, were also probed by Southern hybridization for the presence of the expected DNA structure.

**Southern hybridization.** We performed Southern hybridizations (33) using a probe labeled with digoxigenin conjugated wasC_G and wasC_R primed chain elongation products. This probe was synthesized by PCR as described above, except for the substitution of digoxigenin-dUTP (Boehringer Mannheim) for dTTP. For hybridizations, DNA extracted from the wild-type *B. bronchiseptica* 9.73 and from the *B. bronchiseptica* wasC mutant was digested and transferred to nitrocellulose filters (Hybond N; Amersham), as described by Chomczynski (11). We then hybridized the digoxigenin-labeled DNA probe to the membranes under the conditions specified by the manufacturer and, after blocking nonspecific binding sites, exposed the reacted strips to an antibody against the digoxi- gen ligand (Boehringer Mannheim). In order to visualize the positive bands, the final color reaction was initiated at alkaline pH by the addition of colorless X-phosphate (Boehringer Mannheim) plus tetrazolium blue.

**LPS extraction and SDS-PAGE.** Cells grown at 36°C in Stainer Scholte medium were centrifuged (10,000 × g, 15 min, 4°C) and washed twice in distilled water. After adjusting the bacterial concentration, we extracted the LPS either by the hot phenol-water method (46) along with the modifications previously described (45), or by boiling for 10 min in an aqueous solution of 0.5% (wt/vol) SDS (47). The LPS suspensions were run on 8% (35) or 10% (37) polyacrylamide–SDS gradient gels. Electrophoresis was performed at room temperature and constant voltage, with molecular weights being estimated by means of the Pharmacia calibration kit. After electrophoresis, the proteins were trans- ferred to nitrocellulose membranes (Hybond N; Super: Amersham) and incubated with either a 1:10,000 dilution of hamster polyclonal immune serum directed against pertactin or a 1:5,000 dilution of mouse polyclonal serum directed against adenylate cyclase or filamentous hemagglutinin of *B. bronchiseptica*. Mouse serum (1:500) collected 10 months after infection with wild-type *B. bronchiseptica* 9.73 was also assayed to test its reactivity against LPS isolated from either *B. bronchiseptica* 9.73 or *B. bronchiseptica* wasC mutants. In all cases, we used alkaline phosphatase–linked sheep anti-mouse immunoglobulins to detect the presence of immune complexes.

To obtain the sera, the virulence factors were purified and subsequently inoculated into BALB/c mice as previously described (19, 20).

**Complementation analysis.** Parental strain *B. bronchiseptica* 9.73 DNA was partially digested with EcoRI and BamHI. The EcoRI- and BamHI-digested fragments were ligated into pBBTc (8), digested with EcoRI and BamHI, and introduced into competent *E. coli* S17-1 by transformation. Transformants were grown on LB containing tetracycline (6 μg ml⁻¹). Conjugation incubations were performed in BGA plus 10 mM MgCl₂, using the *B. bronchiseptica* wasC (Sm, Km) mutant as the recipient and *E. coli* S17-1 containing the recombinant replicative plasmid (Tc) as the donor and thereafter selected on BGA plus tetracycline and streptomycin.

We then analyzed the detergent sensitivity of the resultant colonies of transconjugants on Stainer-Scholte solid medium supplemented with 0.02% (wt/vol) SDS. From an SDS-resistant colony, recombinant plasmids, hereafter referred to as pBB3FS, were isolated. The presence of the wasC gene in such plasmids was analyzed by PCR and Southern hybridization.

To clone the wasC gene, the whole gene was amplified by PCR from recombinant plasmid pBB3FS using primers totalwasC_F (5'-TGGC GAA TTC CCA GGA TGT CCA TGA G-3') and totalwasC_R (5'-GCA TGC ACC CAG GAA GAA TTC C-3'). The 1.529-bp PCR product was ligated first into pUC T-Easy (Promega) and then into pBBTc. The last recombinant plasmid was introduced into competent *E. coli* S17-1 by transformation, and the transformants were grown on LB containing tetracycline (6 μg ml⁻¹). Conjugation incubations were performed as described above using the *B. bronchiseptica* wasC (Sm, Km) mutant as the recipient and *E. coli* S17-1 containing the recombinant replicative plasmid (Tc) as the donor. The transconjugants were thereafter selected on BGA plus tetracycline (18 μg ml⁻¹) and streptomycin (200 μg ml⁻¹). We then analyzed the detergent sensitivity of transconjugants on Stainer Scholte solid medium supplemented with 0.02% (wt/vol) SDS and the electrophoretic profile in SDS-PAGE.

**Marine respiratory infection model.** Female BALB/c mice 3 to 4 weeks of age were used as a model of in vivo respiratory infection by *B. bronchiseptica*. Bacteria grown on BGA were resuspended and adjusted to approximately 10⁹ CFU ml⁻¹ in phosphate buffered saline (PBS). Fifty microflters of bacterial suspension was delivered intranasally to each mouse via an air displacement pipette. At different times postinoculation, three mice from each group were sacrificed, and their lungs were removed aseptically. Lungs were homogenized in PBS, and appropriate dilutions were plated onto BGA to determine the number of viable bacteria present in the lungs. All the experiments were repeated three times and gave consistent results.

**Tissue culture and determination of bacterial adherence and persistence.** The human alveolar cell line A549 (ATCC CL193) was used in this study. Cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco Laboratories) supplemented with 10% fetal calf serum, streptomycin (100 μg ml⁻¹), and ampicillin (100 μg ml⁻¹) to 70 to 80% confluence. Twenty-four well Nunclon Delta tissue culture plates (Nunc, Roskilde, Denmark) were seeded with approximately 8 ×
10^4 cells per well 18 h before the assay. For adhesion assays, cells were seeded on glass coverslips previously placed in the selected wells.

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Bacterial strains (either wild type or mutant) were grown for 16 h on SS medium, washed, and suspended in Dubelco’s modified Eagle’s medium supplemented with 10% fetal calf serum without antibiotics to an optical density of 0.5 at 650 nm. Then 8 x 10^8 bacteria in 0.5 ml were added to the wells (ratio of bacteria to cells, 100:1) and centrifuged onto adherent cells at 300 x g. After 2 h at 37°C in 5% CO₂, the monolayers were washed at least five times with PBS (pH 7.2). Then 100 ml of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, streptomycin (100 µg ml⁻¹), and ampicillin (100 µg ml⁻¹) was added. The medium was replaced with 0.5 ml of complete medium containing 100 g ml⁻¹ of polymyxin B and then incubated for 1 h at 37°C in 5% CO₂ to kill extracellular bacteria (9). When B. bronchiseptica alone was treated with this level of polymyxin B, 99.999% of the bacteria were killed over a 1-h period.

Following incubation, the polymyxin B was removed by extensive washing and replaced by 0.5 ml of Dubelco’s modified Eagle’s medium supplemented with 10% fetal calf serum, streptomycin (100 µg ml⁻¹), and ampicillin (100 µg ml⁻¹). The medium was changed every 24 h. At selected time periods, cells containing viable intracellular organisms were recovered from the trypan-treated and stripped monolayers. Trypan blue dye exclusion was used to check eukaryotic cell viability. Intracellular bacteria were counted by plating appropriate dilutions onto BGA. The number of CFU per alveolar cell was then calculated. Each strain was tested in triplicate.

Statistical analysis. Means and standard deviations were calculated from log_{10}-transformed numbers of CFU. Differences between means were assessed by two-tailed Student’s t tests, with significance accepted at the P < 0.05 level.

RESULTS

Cloning of an internal region of the waaC gene of B. bronchiseptica. We amplified a defined 200-bp internal region of the waaC gene of B. bronchiseptica by PCR. By DNA sequencing, we observed that the amplified product reflected a faithful replication of the 200-bp region of the gene spanned by the 14-base stretches complementary to the two primers (waaC and waaC_er), according to previously published data (GenBank). This sequence within the waaC gene of B. bronchiseptica 9.73, in turn, exhibits 97% identity with B. bronchiseptica cosmid BbgLP31 (AJ007747), 97% with the homologous region from the B. pertussis LPS biosynthesis locus (X90711), and 54% identity with the corresponding Escherichia coli heptosyl I transferase waaC gene (AF019746).

We generated a deep rough mutant by effecting a site-specific integration through conjugation with E. coli containing the recombinant plasmid pK18mob waaC. The recombination strategy used was based on site-specific recombination of the nonreplicative vector pK18mob. The internal 200-bp DNA fragment of the waaC coding region used in this strategy was obtained by PCR using primers waaC and waaC_er, as indicated in Materials and Methods. Panel B shows the Southern blot analysis that confirmed the genetic structure of the mutant B. bronchiseptica LP39. Total DNA from B. bronchiseptica 9.73 and B. bronchiseptica LP39 was digested with EcoRI and probed with the 200-bp waaC PCR product labeled with digoxigenin.
FIG. 2. SDS-PAGE profiles and immunoblot analyses of phenol-water-extracted LPS from wild-type *B. bronchiseptica* and *B. bronchiseptica waaC* mutant LP39. (A) Silver-stained SDS-PAGE (8 to 25% [wt/vol]) of LPS extracted from wild-type *B. bronchiseptica* in virulent phase (lane 1) and from a waaC mutant in virulent culture conditions (lane 2). (B) Immunoblots of the SDS-PAGE gel shown in panel A. The gel blot was exposed to mouse antiserum obtained 10 months after infection with wild-type *B. bronchiseptica* as the primary antibody. LPS samples corresponded in all cases to material extracted from approximately 1 mg (wet weight) of bacterial cells. KDO, 3-deoxy-d-manno-octulosonic acid.

FIG. 3. Semiquantitative analysis of virulence determinant expression in wild-type *B. bronchiseptica* and in the *B. bronchiseptica waaC* mutant *B. bronchiseptica* LP39 by immunoblotting. (A) SDS-PAGE (8 to 25% [wt/vol]) of bacterial lysates corresponding to 10^8 CFU of wild-type *B. bronchiseptica* in avirulent phase (lane 1), *B. bronchiseptica* in virulent phase (lane 2), and *B. bronchiseptica waaC* mutant LP39 in virulent culture conditions (lane 3). Proteins were stained overnight in an aqueous solution of Coomassie brilliant blue R250 (0.2% [wt/vol]). (B to D) Western blots of the SDS-PAGE gel shown in panel A. Antisera against adenylate cyclase (B), filamentous hemagglutinin (C), and pertactin (D) were used.

**Southern transfer analysis.** We digested DNA from the wild-type strain and from the *B. bronchiseptica waaC* mutant with EcoRI, an enzyme that recognizes a single site in the nonrecombined pK18mob suicide plasmid, and then separated the resulting total DNA digests by agarose gel electrophoresis. We then exposed the electrophoresed gels to a waaC-specific digoxigenin-DNA probe. Subsequent treatment of the labeled gels with digoxigenin-specific antibodies indicated probe hybridization to a single EcoRI fragment within the samples from the parental strain, with this species being replaced by two fragments of smaller size in the waaC mutants (Fig. 1B). As expected, we observed that the sum of the sizes of the two waaC fragments (approximately 4,200 and 6,300 kb) corresponded to the size of the parental-strain single fragment (approximately 6,800 kb) plus the size of the vector (approximately 3,700 kb). This result confirmed the correct insertion of the waaC recombinant plasmid into the *B. bronchiseptica* chromosome after the conjugation event.

**LPS electrophoresis and immunoblotting.** We analyzed the LPS phenotype of *B. bronchiseptica* LP39 by SDS-PAGE using silver staining (Fig. 2A). The LPS profile from *B. bronchiseptica* LP39 exhibited no O-antigen band; moreover, only a single LPS band was present, which migrated considerably faster than the corresponding wild-type species. These electrophoretic properties are consistent with the deep rough phenotype. Furthermore, when we carried out immunoblots of these LPS species using sera from *B. bronchiseptica*-infected mice, we observed that the wild-type LPS was serologically recognized, whereas the mutant LPS was not (Fig. 2B).

**Characterization of expression of virulence determinants in *B. bronchiseptica* LP39.** In order to evaluate if *B. bronchiseptica* LP39 still had the ability to respond to well-known virulence phase modulatory influences such as low temperature and sulfa, we incubated both wild-type and recombinant bacteria at 25°C in either BGA alone or BGA supplemented with 40 mM MgSO_4_ and used hemolytic activity as a marker of virulence. After 48 h of exposure to sulfate under these conditions, both the wild-type strain and the *B. bronchiseptica* LP39 mutant shifted from a virulent to an avirulent state (data not shown). At 36°C, both strains had approximately the same diameter of hemolysis in BGA. These findings indicate that the bvgAS two-component regulatory system remains functional in the waaC mutants.

We then performed a semiquantitative analysis of the expression of virulence determinants by Western blotting, using mouse polyclonal immune sera directed against purified adenylate cyclase, pertactin, and filamentous hemagglutinin (Fig. 3). Although a decrease in both filamentous hemagglutinin and pertactin could be observed in the *B. bronchiseptica* LP39 mutant lysates, in the case of pertactin the decrease in intensity was especially notable (Fig. 3).

**Complementation of the waaC mutant phenotype.** The *B. bronchiseptica* LP39 mutant displayed an alteration in its LPS profile upon SDS-PAGE and an enhanced sensitivity to SDS. To rule out the possibility that these phenotypes were due to some uncontrolled polar effect, we obtained a clone that complements in *trans* the defect in LPS caused by the insertion of the pK18mob::waaC recombinant plasmid. The DNA region contained in plasmid pJB3FS complemented both the alteration in the SDS-PAGE profile (Fig. 4) and the SDS sensitivity (not shown) of the *B. bronchiseptica* LP39 mutant strain. In addition, we were able to amplify a DNA fragment with the expected size (1.529 bp) using the *totalwaaC* and *totalwaaC* primers.

**Survival of *B. bronchiseptica* LP39 mutant within the murine respiratory tract.** Nonlethal doses of the *B. bronchiseptica* LP39 mutant strain were administered to BALB/c mice intra-
of infection, we tried to overcome the colonization deficiency of *B. bronchiseptica* LP39 by performing coinoculation experiments in which the parental and mutant strains were inoculated with a delay of 1 h. In this kind of experiment, the parental strain inoculated first could suppress the colonization deficiency of *B. bronchiseptica* LP39, as demonstrated for other microorganism-host interactions (18). The parental and *B. bronchiseptica* LP39 colonies were distinguished by differential antibiotic sensitivity; the parental strain was Sm’, while *B. bronchiseptica* LP39 was Sm’ and Km’. Again, *B. bronchiseptica* LP39 was cleared within 5 days following the infection (data not shown).

To determine whether the first-inoculated strain precludes the colonization of the second one, we performed an independent coinoculation experiment in which the second inoculation was performed with a labeled parental strain carrying plasmid pGB5P1 as a marker, to differentiate it from the first inoculum. In this case, the colonization kinetics of labeled *B. bronchiseptica* exhibited the observed pattern of parental strain infection (data not shown).

We also varied the delay (5 to 20 days) between the first and second inoculum. Interestingly, we did not detect significant changes in the results obtained with either the mutant or the labeled wild-type strains even in those experiments, in which the second inoculation was performed as late as 20 days after the first one. These data seem to indicate that under our experimental conditions, no protection was induced after 20 days of infection.

**Adhesion and intracellular survival of parental and *B. bronchiseptica* LP39 mutant strains.** In vitro assays demonstrated that the adherence of *B. bronchiseptica* LP39 to human pulmonary epithelial cells (5 ± 4 bacteria/epithelial cell) was significantly lower than that exhibited by the parental strain (62 ±

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**FIG. 4.** SDS-PAGE (18% [wt/vol]) analysis showing the genetic complementation of the *waaC* mutant *B. bronchiseptica* LP39 with the genomic library of the parental strain. Each well contained LPS extracted from the wild-type *B. bronchiseptica* strain (lane 1), *waaC* mutant *B. bronchiseptica* LP39 carrying the pJB3FS recombinant plasmid (lane 2), and *waaC* mutant *B. bronchiseptica* LP39 (lane 3). The samples were obtained from around 1 to 2 mg (wet weight) of bacterial cells using EDTA and polymyxin B as described in Materials and Methods. The positions of the main LPS components in the gel are indicated to the left. KDO, 3-deoxy-d-manno-octulosonic acid.

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**FIG. 5.** In vivo persistence of wild-type *B. bronchiseptica* 9.73 and *B. bronchiseptica* *waaC* mutant LP39 in a murine respiratory model. Lungs were extracted at different times, and the number of viable bacteria per lung was determined. The results represent the means ± standard deviation of three independent experiments.
Infect. Immun.

A

B

FIG. 6. Phase-contrast micrographs of the adherence of *B. bronchiseptica* strains to human A549 alveolar epithelial cells, used in a standard adherence assay with (A) the parental strain and (B) the *waaC* mutant *B. bronchiseptica* LP39. Magnification, 1,000×. Panels are representative of one to three independent experiments.

25 bacteria/epithelial cell) (Fig. 6). The *waaC* mutant also showed a lower invasion rate than the parental strain. This result was more likely to be caused by the reduced number of attached bacteria than by a defect in bacterial invasive ability. However, this last possibility could not be ruled out in our system.

For both bacteria, intracellular survival showed the same profile over time. A decrease in bacterial viability was observed

FIG. 7. Intracellular survival of *B. bronchiseptica* wild-type strain and *B. bronchiseptica* *waaC* mutant LP39 in the human alveolar epithelial cell line A549. At selected time periods, the number of CFU per alveolar cell was determined. The data represent the means ± standard deviation of three independent experiments.
during the first days postinvasion (Fig. 7), after which both the mutant and the parental strain showed a constant increase in the number of live bacteria per eukaryotic cell during the course of the experiment.

**DISCUSSION**

LPS has been shown to be important for virulence in many bacterial pathogens, and its contribution to the infection cycle by a variety of mechanisms, including antigenic variation, molar mimicry, and induction of blocking antibodies, has been proposed (30, 31, 43). In *Bordetella*, however, the role of LPS during infection has been little investigated. In recent studies, Spears et al. (38) isolated and characterized in vitro and in vivo two LPS wlb mutants of *Bordetella avium*. These LPS mutants were affected in their ability to colonize turkey trachea. In addition, wlb deletion mutants of three other species of *Bordetella*, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, were also described. In that work, the authors showed that the biosynthesis of a full-length LPS molecule by these three species of *bordetellae* is essential for the expression of full virulence in mice. West et al. (46) described an LPS mutant of *B. bronchiseptica* resulting from an insertion in the phosphoglucomutase-encoding gene (BB7865pgm). This mutant showed a diminished ability to survive either in vitro or in vivo following intranasal infection of mice. However, none of these reports described the in vitro or in vivo role of deep rough LPS, a phenotype observed during human *B. bronchiseptica* infection, in the interaction with host cells.

Here we have approached the construction of a waaC mutant of *B. bronchiseptica* on the basis of genetic alterations that in other gram-negative bacteria have been shown to produce a deep rough LPS structure compatible with bacterial viability. The resulting waaC mutation of *B. bronchiseptica* was not lethal, and the altered bacteria were still able to produce detectable levels of the major virulence factors adenylate cyclase, filamentous hemagglutinin, and pertactin (Fig. 3) and responded to well-known modulators of the bvgAS system, such as low temperature and sulfate ion. These observations indicate that the bvgAS sensor and response regulator functions remain unaltered even within the context of a deep rough LPS mutation.

We observed that the mutation in the waaC gene of *B. bronchiseptica* reduced the expression of pertactin and to a lesser extent of filamentous hemagglutinin. Since all of these proteins are related to the outer membrane, the alteration of this cellular compartment by changes in the structure of the LPS could explain the observed protein reduction levels. This decrease in virulence factors might favor the persistence of the bacteria within the host during chronic manifestations of *B. bronchiseptica* infection. Indeed, the production of bvg-activated gene products may be a disadvantage in terms of the intracellular survival of the bacteria, since the expression of certain virulence factors is highly toxic to the infected eukaryotic cells (1, 5, 17).

Interestingly, the LPS from the mutant *B. bronchiseptica* LP39 was immunologically different from the wild-type LPS. Accordingly, the waaC mutation that exhibits no O-antigen determines a complete loss of LPS serologic reactivity (Fig. 2B), including that of the higher-mobility component, which is strongly reactive within the LPS of the parental strain (Fig. 2B). These results resemble those observed during chronic infections in which sera that still react with intact *B. bronchiseptica* LPS isolated from early stages of infection failed to immunologically recognize the deep rough LPS of bacteria isolated from the same human infections (data not shown).

Our results from the murine respiratory infection model clearly demonstrated that the deep rough phenotype of the LPS diminishes *B. bronchiseptica*’s ability to colonize mice. The *B. bronchiseptica* LP39 mutant was cleared from lungs within 5 days, whereas the parental strain persisted for at least 30 days postinfection (Fig. 5). This decrease in colonization ability could not be overcome by coinfection experiments with the parental strain, indicating that the waaC phenotype cannot be complemented with wild-type LPS, which has been shown to be released into the surrounding medium (25).

These data point out that the *B. bronchiseptica* LP39 mutant, either alone or in combination with the wild-type strain, is unable to colonize mouse lungs and suggest that the observed deep rough LPS phenotype isolated from chronic infections (20, 21) would not come from mutants that infected the host from the beginning. By contrast, these deep rough bacteria would have arisen from already established infections.

In agreement with this inability to colonize, the *B. bronchiseptica* LP39 mutant attaches to pulmonary cells less efficiently than the wild-type strain. However, persistence kinetics of both strains within the eukaryotic cells showed similar patterns over the time period studied. Figure 7 shows an initial decrease in bacterial survival, which could suggest a lag period in which the bacteria have to adapt to environmental conditions, followed by a significant increase in the number of live bacteria per eukaryotic cell over time.

These results suggest that both strains of *B. bronchiseptica* are able not only to survive but also to replicate inside pulmonary cells. After 14 days, significant eukaryotic cell death was detected. A similar cell death rate was found in both infected and noninfected (control) cells, indicating that death was not caused by *B. bronchiseptica* infection. Due to these circumstances, in vitro studies could not be performed for more than 2 weeks, which excludes the possibility of a long-term survival comparison of the strains.

According to our results, the truncated form of *B. bronchiseptica* LPS isolated from chronic human infections and animal infections (20, 21, 29) can only be explained if we assume that they derive from smooth forms which were able to colonize. The concomitant modification of the expression pattern of the principal virulence factors and the complete loss of LPS serologic reactivity in the waaC mutant underscore the idea that structural changes that could occur during infection help bacteria to persist within the host.

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