INFECTION AND IMMUNITY, Apr. 2002, p. 1791–1798 Vol. 70, No. 4
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.4.1791–1798.2002

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

Federico Sisti,[1] Julieta Fernández,[1,2] María Eugenia Rodríguez,[2] Antonio Lagares,[1] Nicole Guiso,[3] and Daniela Flavia Hozbor[1,2]*

Instituto de Bioquímica y Biología Molecular[1] 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 Cedex 15, France[3]

Received 1 August 2001/Returned for modification 1 November 2001/Accepted 10 January 2002

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*-acetylgalactosaminuronic 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 *bvg* (*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-

* Corresponding author. Mailing address: Centro de Investigación y Desarrollo en Fermentaciones Industriales, CINDEFI, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calles 47 y 115, 1900 La Plata, República Argentina. Phone: 54-221-483-3794. Fax: 54 221 4833794. E-mail: hozbor@nahuel.biol.unlp.edu.ar.

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 *waaC* 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 waaC* 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 200 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 replated 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 pGB5P1 (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 Alisson 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 2×10^{10} CFU ml^{-1}, diluted in Laemmli buffer (28), and boiled for 15 min.

PCR and recombinant DNA techniques. Based on previously reported consensus sequences (2, 3, 7, 16, 36) (*B. bronchiseptica* cosmid BbLPS1 AJ007747), we designed two single-stranded oligonucleotide primers, $waaC_{pf}$ (5′-TTCAIC AGCCCCTG-3′, where I is inosine) and $waaC_{pr}$ (5′-CCAGATTGACGGGT-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 *waaC* gene. This PCR product was cloned into the shuttle plasmid pGem T-Easy (Ap^r, *lacZ*: Promega). After ligation, a recombinant fragment from this plasmid was released with *Eco*RI and cloned into the *Eco*RI site of the *Bordetella* suicide plasmid pK18mob (Km^r) (35).

We performed conjugation incubations in BGA plus 10 mM $MgCl_2$, using *B. bronchiseptica* 9.73 Sm^r as the recipient and *E. coli* S17-1 containing the recombinant suicide plasmid as the donor, and thereafter colonies were selected for single genetic crossovers on kanamycin plus streptomycin. We then analyzed the detergent sensitivity of the resultant colonies of transconjugants on Stainer-Scholte solid medium (agar 1.5% [wt/vol]; Sigma Chemical Co.) supplemented with 0.02% (wt/vol) sodium dodecyl sulfate (SDS). Genomic DNAs from the SDS-sensitive colonies, hereafter referred to as *B. bronchiseptica waaC* 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 $waaC_{pf}$- and $waaC_{pr}$-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 waaC* mutant was digested and transferred to nitrocellulose strips (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 digoxigenin 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 (24), or by affinity chromatography (41). The procedure employed is indicated in the legends to the figures. In both cases the isolated LPS was solubilized by heating at 100°C for 5 min in Laemmli sample buffer (28). We then applied the LPS suspensions to SDS gels. Gel acrylamide concentrations are indicated in the legends to the figures.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed at room temperature and constant voltage. The LPS was visualized by the Bio-Rad silver-staining technique.

Detection of adenylate cyclase, pertactin, filamentous hemagglutinin, and LPS in Western immunoblots. Cells corresponding to 10^8 CFU of the *B. bronchiseptica* parental strain and the *B. bronchiseptica waaC* mutant were treated with Laemmli sample buffer (28), and the extracts were run on 8 to 25% (wt/vol) 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 transferred to nitrocellulose membranes (Hybond C-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 mice polyclonal immune 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 waaC* mutants. In all cases, we used alkaline phosphatase-labeled 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 *Eco*RI and *Bam*HI. The *Eco*RI- and *Bam*HI-digested fragments were ligated into pJB3Tc (8), digested with *Eco*RI and *Bam*HI, and introduced into competent *E. coli* S17-1 by transformation. Transformants were grown on LB containing tetracycline (6 μg/ml). Conjugation incubations were performed in BGA plus 10 mM $MgCl_2$, using the *B. bronchiseptica waaC* (Sm^r, Km^r) mutant as the recipient and *E. coli* S17-1 containing the recombinant replicative plasmid (Tc^r) 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 pJB3FS, were isolated. The presence of the *waaC* gene in such plasmids was analyzed by PCR and Southern hybridization.

To clone the *waaC* gene, the whole gene was amplified by PCR from recombinant plasmid pJB3FS using primers $totalwaaC_f$ (5′-TGC GAA TTC CCA GCA TGT CGC TGA G-3′) and $totalwaaC_r$ (5′-GCA TGC ACC CAG ACC GAA TTC C-3′). The 1,529-bp PCR product was ligated first into pGem-T-Easy (Promega) and then into pJB3Tc. 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 waaC* (Sm^r, Km^r) mutant as the recipient and *E. coli* S17-1 containing the recombinant replicative plasmid (Tc^r) 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.

Murine 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 10^7 CFU ml^{-1} in phosphate-buffered saline (PBS). Fifty microliters 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 adhesion and persistence. The human alveolar cell line A549 (ATCC CCL185) 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 ×

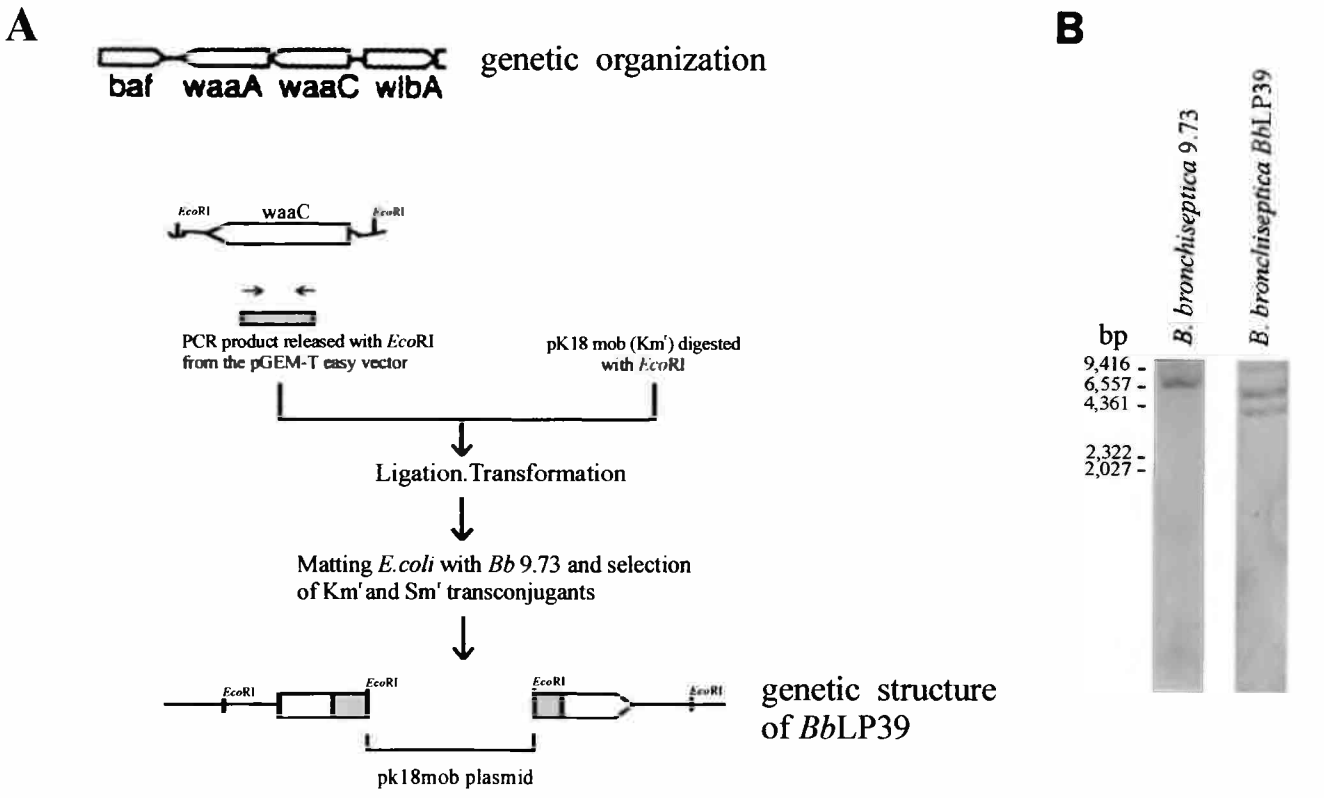

FIG. 1. Construction of a *waaC* defective mutant in *B. bronchiseptica* 9.73. Panel A shows the mutagenesis strategy used, based on site-specific recombination of the nonreplicative vector pK18mob. The internal 200-bp DNA fragment of the *waaC* coding region used in this strategy was obtained by PCR using primers $waaC_{pf}$ and $waaC_{pr}$, 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 *Eco*RI and probed with the 200-bp *waaC* PCR product labeled with digoxigenin.

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.

Bacterial strains (either wild type or mutant) were grown for 16 h on SS medium, washed, and suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum without antibiotics to an optical density of 0.5 at 650 nm. Then 8×10^6 bacteria in 0.5 ml were added to the wells (ratio of bacteria to cells, 100:1) and centrifuged onto adherent cells at $300 \times g$. After 2 h at 37°C in 5% CO_2, the monolayers were washed at least five times with PBS (pH 7.2).

To determine bacterial adhesion, the cells grown on glass coverslips with attached bacteria were fixed in methanol, and the staining of cells and bacteria was done with crystal violet (0.07% in water). Examination was carried out using phase-contrast microscopy at 1,000× magnification. Approximately 50 cells were examined to calculate the number of adherent bacteria per epithelial cell. All experiments were done at least three times in duplicate.

To determine bacterial survival within the alveolar cell, after the 2-h incubation period and the extensive washing described above, the monolayers were further incubated for 3 h at 37°C in 5% CO_2 to allow bacterial invasion. The medium was then replaced with 0.5 ml of complete medium containing 100 μg of polymyxin B ml^{-1} and then incubated for 1 h at 37°C in 5% CO_2 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 Dulbecco'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 trypsin-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 ($waaC_{pf}$ and $waaC_{pr}$), 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 BbLPS1 (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* (Fig. 1A). Since detergent sensitivity should also become altered in such a deep rough mutant (36), we confirmed that the resulting streptomycin- and kanamycin-resistant genomic transconjugants were

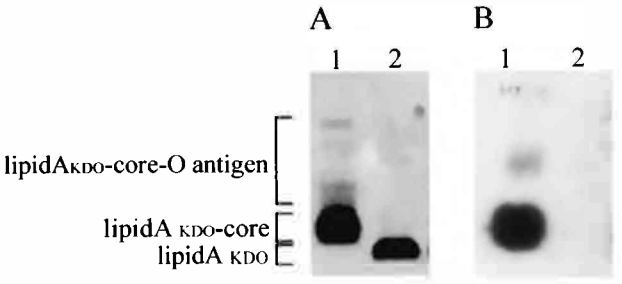

FIG. 2. SDS-PAGE profiles and immunoblot analyses of phenol-water-extracted LPS from wild-type *B. bronchiseptica* and *B. bronchiseptica waaC* mutant LP39. (A) Silver-stained SDS-PAGE (8 to 25% [wt/vol]) of LPS extracted from wild-type *B. bronchiseptica* in virulent phase (lane 1) and from a *waaC* 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.

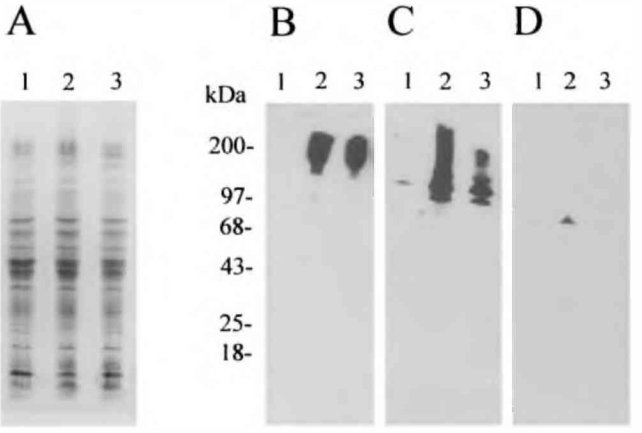

FIG. 3. Semiquantitative analysis of virulence determinant expression in wild-type *B. bronchiseptica* and in the *B. bronchiseptica waaC* mutant *B. bronchiseptica* LP39 by immunoblotting. (A) SDS-PAGE (8 to 25% [wt/vol]) of bacterial lysates corresponding to 10^8 CFU of wild-type *B. bronchiseptica* 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.

unable to grow on Stainer-Scholte solid medium supplemented with 0.02% (wt/vol) SDS.

Southern transfer analysis. We digested DNA from the wild-type strain and from the *B. bronchiseptica waaC* mutant with *Eco*RI, 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 *Eco*RI 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.

For the following studies, we selected one of the confirmed *waaC* mutants, hereafter referred to as the *B. bronchiseptica* LP39 mutant.

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 sulfate anion, 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 those 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*$_f$ and *totalwaaC*$_r$ 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-

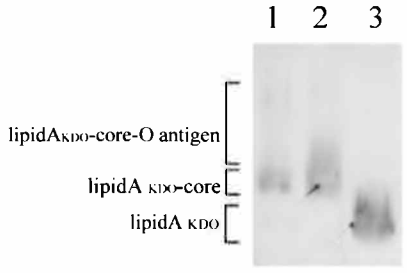

FIG. 4. SDS-PAGE (18% [wt/vol]) analysis showing the genetic complementation of the *waaC* mutant *B. bronchiseptica* LP39 with the genomic library of the parental strain. Each well contained LPS extracted from the wild-type *B. bronchiseptica* strain (lane 1), *waaC* mutant *B. bronchiseptica* LP39 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.

nasally, and the number of CFU present in the lungs was measured at different time points following infection (Fig. 5). Although the wild-type strain showed a classic pattern of infection (19), the mutant strain was unable to survive, being effectively cleared within 5 days. Wild-type bacteria could still be recovered from the infected mice's lungs 30 days after inoculation. This deficiency of *B. bronchiseptica* LP39 in mouse colonization was completely restored by complementation with the DNA region contained in plasmid pJB3FS. In this experiment, *B. bronchiseptica* LP39 harboring the replicative plasmid pJB3FS displayed a lung colonization profile statistically indistinguishable from that of the parental strain.

In order to analyze the behavior of the mutant at late stages 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^r, while *B. bronchiseptica* LP39 was Sm^r and Km^r. 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 ±

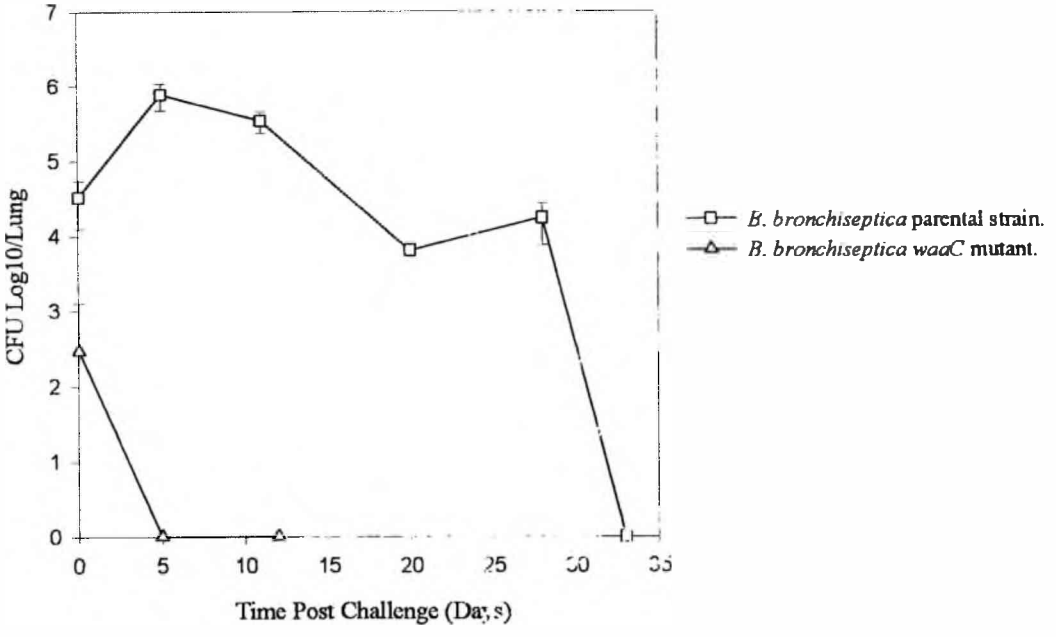

FIG. 5. In vivo persistence of wild-type *B. bronchiseptica* 9.73 and *B. bronchiseptica waaC* mutant LP39 in a murine respiratory model. Lungs were extracted at different times, and the number of viable bacteria per lung was determined. The results represent the means ± standard deviation of three independent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

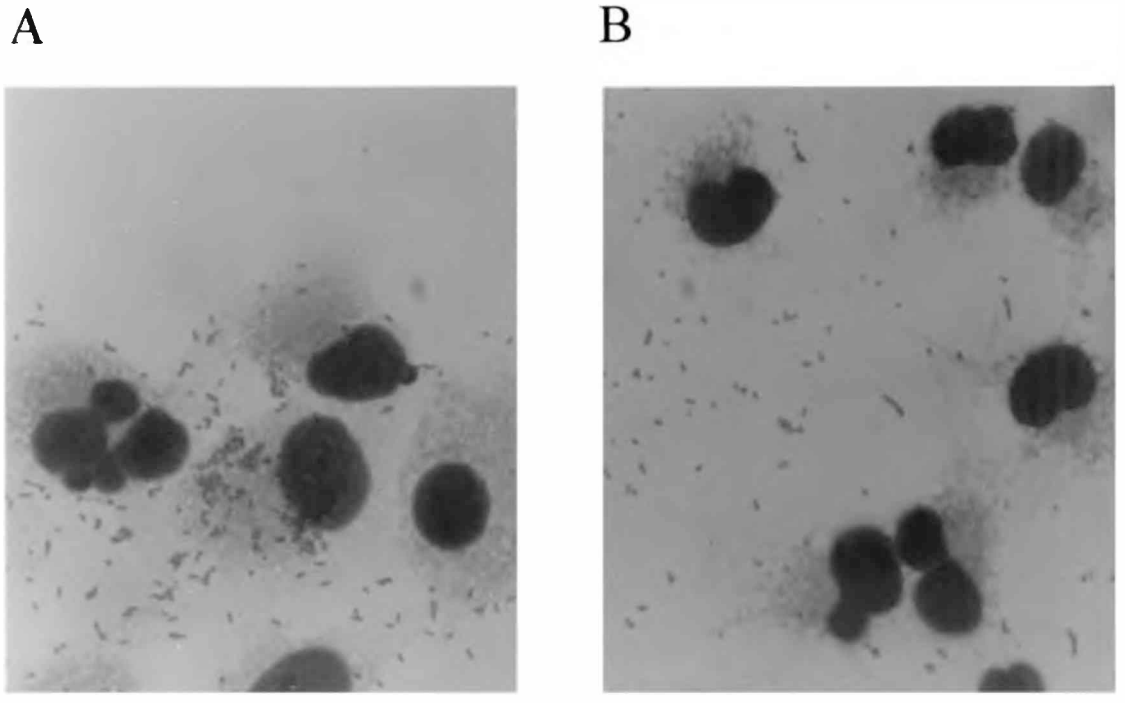

FIG. 6. Phase-contrast micrographs of the adherence of *B. bronchiseptica* strains to human A549 alveolar epithelial cells, used in a standard adherence assay with (A) the parental strain and (B) the *waaC* mutant *B. bronchiseptica* LP39. Magnification, 1,000×. Panels are representative of one to three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

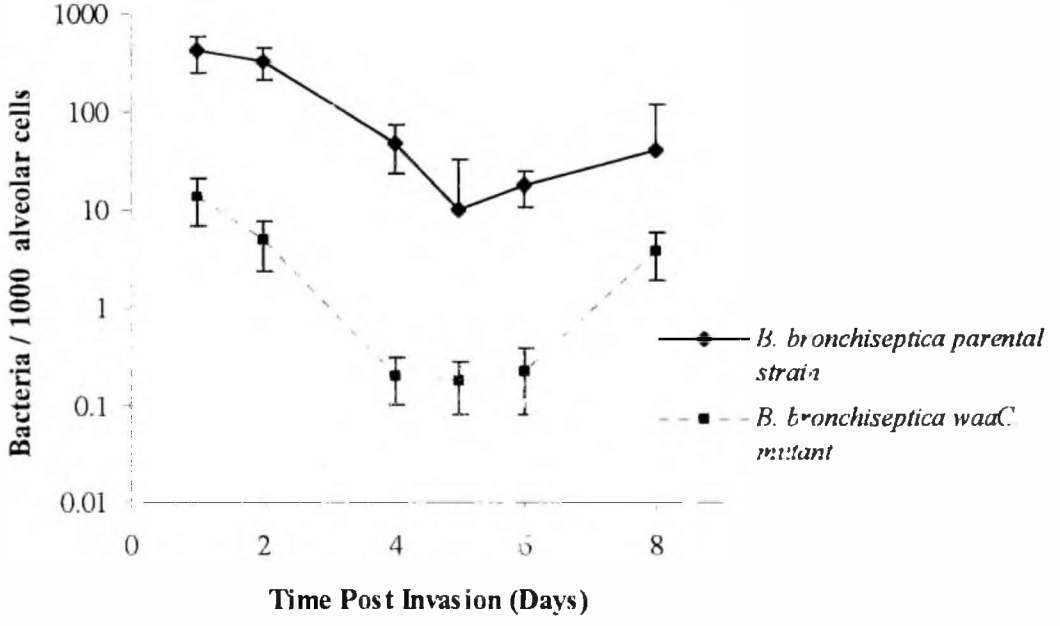

FIG. 7. Intracellular survival of *B. bronchiseptica* wild-type strain and *B. bronchiseptica waaC* mutant LP39 in the human alveolar epithelial cell line A549. At selected time periods, the number of CFU per alveolar cell was determined. The data represent the means ± standard deviation of three independent 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, molecular 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.

ACKNOWLEDGMENTS

This work was supported by grants SECYT-PICT97 01-00000-00623 and IFS-B/2672-2 to D.F.H., B/2993-1 to M.E.R., CONICET (Argentina)— INSERM (France), and partially by CICBA and CONICET (Argentina). D.F.H. is a member of the Scientific Career of CIC. M.E.R., F.S., J.F., and A.L. are members of CONICET.

We are grateful to Donald F. Haggerty and Christina McCarthy for critical reading and editing of the manuscript.

REFERENCES

1. **Abramson, T., H. Kedem, and D. A. Relman.** 2001. Proinflammatory and proapoptotic activities associated with *Bordetella pertussis* filamentous hemagglutinin. Infect. Immun. **69:**2650–2658.
2. **Allen, A., and D. Maskell.** 1996. The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in *Bordetella pertussis*. Mol. Microbiol. **19:**37–52.
3. **Allen, A. G., R. M. Thomas, J. T. Cadisch, and D. J. Maskell.** 1998. Molecular and functional analysis of the lipopolysaccharide biosynthesis locus *wlb* from *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. Mol. Microbiol. **29:**27–38.
4. **Arico, B., V. Scarlato, D. Monack, S. Falkow, and R. Rappuoli.** 1991. Structural and genetic analysis of the *bvg* locus in *Bordetella* species. Mol. Microbiol. **5:**481–491.
5. **Banemann, A., and R. Gross.** 1997. Phase variation affects long-term survival of *Bordetella bronchiseptica* in professional phagocytes. Infect. Immun. **65:**3469–3473.
6. **Banemann, A., H. Deppisch, and R. Gross.** 1998. The lipopolysaccharide of *Bordetella bronchiseptica* acts as a protective shield against antimicrobial peptides. Infect. Immun. **66:**5607–5612.
7. **Bauer, M., and R. Welch.** 1997. Pleiotropic effects of a mutation in *rfaC* on *Escherichia coli* hemolysin. Infect. Immun. **65:**2218–2224.
8. **Blatny, J. M., T. Brautaset, H. C. Winther-Larsen, K. Haugan, and S. y Valla.** 1997. Construction and use of a versatile set of broad-host-range cloning and expression vectors based on the RK2 replicon. Appl. Environ. Microbiol. **63:**370–379.
9. **Brockmeier, S. L., and K. B. Register.** 2000. Effect of temperature modulation and *bvg* mutation of *Bordetella bronchiseptica* on adhesion, intracellular survival and cytotoxicity for swine alveolar macrophages. Vet. Microbiol. **73:**1–12.
10. **Camprubi, S., J. Tomas, F. Munoa, C. Madrid, and A. Juarez.** 1990. Influence of lipopolysaccharide on external hemolytic activity of *Salmonella typhimurium* and *Klebsiella pneumoniae*. Curr. Microbiol. **20:**1–3.
11. **Chomczynski, P.** 1991. One-hour downward alkaline capillary transfer for blotting DNA and RNA. Anal. Biochem. **201:**134–139.
12. **Coote, J. G.** 1992. Structural and functional relationships among RTX toxin determinants of Gram-negative bacteria. FEMS Microbiol. **88:**137–161.
13. **Cotter, P., and J. Miller.** 1997. A mutation in *Bordetella bronchiseptica bvgS* gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg regulated antigens. Mol. Microbiol. **24:**671–685.
14. **Cotter, P. A., M. H. Yuk, S. Mattoo, B. J. Akerley, J. Boschwitz, D. A. Relman, and J. F. Miller.** 1998. Filamentous hemagglutinin of *Bordetella bronchiseptica* is required for efficient establishment of tracheal colonization. Infect. Immun. **66:**5921–5929.
15. **de Kievit, T. R., and J. S. Lam.** 1997. Isolation and characterization of two genes, *waaC* (*rfaC*) and *waaF* (*rfaF*), involved in *Pseudomonas aeruginosa* serotype O5 inner-core biosynthesis. J. Bacteriol. **179:**3451–3457.
16. **Deora, R., H. J. Bootsma, J. F. Miller, and P. A. Cotter.** 2001. Diversity in the *Bordetella* virulence regulon: transcriptional control of a Bvg-intermediate phase gene. Mol. Microbiol. **40:**669–683.
17. **Forde, C. B., X. Shi, J. Li, and M. Roberts.** 1999. *Bordetella bronchiseptica*-mediated cytotoxicity to macrophages is dependent on *bvg*-regulated factors, including pertactin. Infect. Immun. **67:**5972–5978.
18. **Gonzalez, J. E., B. L. Reuhs, and G. C. Walker.** 1996. Low molecular weight EPS II of *Rhizobium meliloti* allows nodule invasion in *Medicago sativa*. Proc. Natl. Acad. Sci. USA **93:**8636–8641.
19. **Gueirard, P., and N. Guiso.** 1993. Virulence of *Bordetella bronchiseptica*: role of adenylate cyclase-hemolysin. Infect. Immun. **61:**4072–4078.
20. **Gueirard, P., C. Weber, A. Le Coustumier, and N. Guiso.** 1995. Human *Bordetella bronchiseptica* infection related to contact with infected animals: persistence of bacteria in host. J. Clin. Microbiol. **33:**2002–2006.
21. **Gueirard, P., K. Le Blay, A. Le Coustumier, R. Chaby, and N. Guiso.** 1998. Variation in *Bordetella bronchiseptica* lipopolysaccharide during human infection. FEMS Microbiol. Lett. **15:**331–337.
22. **Harvill, E. T., P. A. Cotter, M. H. Yuk, and J. F. Miller.** 1999. Probing the function of *Bordetella bronchiseptica* adenylate cyclase toxin by manipulating host immunity. Infect. Immun. **67:**1493–1500.
23. **Harvill, E. T., A. Preston, P. A. Cotter, A. Allen, D. J. Maskell, and J. F. Miller.** 2000. Multiple roles for *Bordetella* lipopolysaccharide molecules during respiratory tract infection. Infect. Immun. **68:**6720–6728.
24. **Hozbor, D., M. E. Rodriguez, A. Samo, A. Lagares, and O. Yantorno.** 1993. Release of lipopolysaccharide during *Bordetella pertussis* growth. Res. Microbiol. **144:**201–209.
25. **Hozbor, D., M. E. Rodriguez, J. Fernández, A. Lagares, N. Guiso, and O. Yantorno.** 1999. Release of outer membrane vesicles from *Bordetella pertussis*. Curr. Microbiol. **38:**273–278.
26. **Jungnitz, H., N. P. West, M. J. Walker, G. S. Chhatwal, and C. A. Guzman.** 1998. A second two-component regulatory system of *Bordetella bronchiseptica* required for bacterial resistance to oxidative stress, production of acid phosphatase, and in vivo persistence. Infect. Immun. **66:**4640–4650.
27. **Khelef, N., H. Sakamoto, and N. Guiso.** 1992. Both adenylate cyclase and hemolytic activities are required by *Bordetella pertussis* to initiate infection. Microb. Pathog. **12:**227–235.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
29. **Le Blay, K., P. Gueirard, N. Guiso, and R. Chaby.** 1997. Antigenic polymorphism of the lipopolysaccharides from human and animal isolates of *Bordetella*. Microbiology **143:**1433–1441.
30. **Preston, A., R. F. Mandrell, B. W. Gibson, and M. A. Apicella.** 1996. The lipooligosaccharides of pathogenic gram-negative bacteria. Crit. Rev. Microbiol. **22:**139–180.
31. **Preston, A., A. G. Allen, J. Cadisch, R. Thomas, K. Stevens, C. M. Churcher, K. L. Badcock, J. Parkhill, B. Barrell, and D. J. Maskell.** 1999. Genetic basis for lipopolysaccharide O-antigen biosynthesis in bordetellae. Infect. Immun. **67:**3763–3767.
32. **Ray, A., K. Redhead, S. Seikirk, and S. Poole.** 1991. Variability in LPS composition, antigenicity and reactogenicity of phase variants of *Bordetella pertussis*. FEMS Microbiol. Lett. **79:**211–218.
33. **Sambrook, J., T. Maniatis, and E. F. Fritsch.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Scarlato, V., B. Arico, A. Prugnola, and R. Rappuoli.** 1991. Sequential and environmental regulation of the virulence genes in *Bordetella pertussis*. EMBO J. **10:**3971–3975.
35. **Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler.** 1994. Small mobilizable multipurpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.
36. **Schnaitman, C. A., and J. Klena.** 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. **57:**655–682.
37. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–791.
38. **Spears, P. A., L. M. Temple, and P. E. Orndorff.** 2000. A role for lipopolysaccharide in turkey tracheal colonization by *Bordetella avium* as demonstrated in vivo and in vitro. Mol. Microbiol. **36:**1425–1435.
39. **Stainer, D., and M. Scholte.** 1971. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63:**211–220.
40. **Stanley, P., P. Diaz, M. J. A. Bailey, D. Gygi, A. Juarez, and C. Hughes.** 1993. Loss of activity in the secreted form of *Escherichia coli* haemolysin caused by an *rfaP* lesion in core lipopolysaccharide assembly. Mol. Microbiol. **10:**781–787.
41. **Valverde, C., D. F. Hozbor, and A. Lagares.** 1997. Rapid preparation of affinity-purified lipopolysaccharide samples for electrophoretic analysis. BioTechniques **22:**230–235.
42. **van den Akker, W.** 1998. Lipopolysaccharide expression within the genus *Bordetella*: influence of temperature and phase variation. Microbiology **144:**1527–1535.
43. **van Putten, J. P.** 1993. Phase variation of lipopolysaccharide directs interconversion of invasive and immuno-resistant phenotypes of *Neisseria gonorrhoeae*. EMBO J. **12:**4043–4051.
44. **Wandersman, C., and S. Létoffé.** 1993. Involvement of the lipopolysaccharide in the secretion of *Escherichia coli* alpha-hemolysin and *Erwinia chrysanthemi* proteases. Mol. Microbiol. **7:**141–150.
45. **Weingart, C. L., G. Broitman-Maduro, G. Dean, S. Newman, M. Peppler, and A. A. Weiss.** 1999. Fluorescent label influences phagocytosis of *Bordetella pertussis* by human neutrophils. Infect. Immun. **67:**4264–4267.
46. **West, N., H. Jungnitz, J. Fitter, J. McArthur, C. Gůzman, and M. Walker.** 2000. Role of phosphoglucomutase of *Bordetella bronchiseptica* in lipopolysaccharide biosynthesis and virulence. Infect. Immun. **68:**4673–4680.
47. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharide. Methods Carbohydr. Chem. **5:**83–91.
48. **Woolfrey, B. F., and J. A. Moody.** 1991. Human infections associated with *Bordetella bronchiseptica*. J. Clin. Microbiol. **4:**243–255.

Editor: R. N. Moore