INFECTION AND IMMUNITY, Oct. 1997, p. 4222–4228 Vol. 65, No. 10
0019-9567/97/$04.00+0

Role of the *Bordetella pertussis* Minor Fimbrial Subunit, FimD, in Colonization of the Mouse Respiratory Tract

CECILE A. W. GEUIJEN,[1] ROB J. L. WILLEMS,[1] MAARTEN BONGAERTS,[1] JANETTA TOP,[1] HENK GIELEN,[2] AND FRITS R. MOOI[1]*

Research Laboratory for Infectious Diseases[1] and Animal Science Department,[2] National Institute of Public Health and Environment, 3720 BA Bilthoven, The Netherlands

Received 20 May 1997/Returned for modification 16 July 1997/Accepted 31 July 1997

Bordetella pertussis **fimbriae are composed of a major subunit, Fim2 or Fim3, and the minor subunit FimD. Using immunoelectron microscopy, we provide evidence that FimD is located at the fimbrial tip. The role of FimD in colonization of the mouse respiratory tract was studied by using two fimbrial mutants: a mutant completely devoid of fimbriae (designated FimD$^-$) and a mutant devoid of the major fimbrial subunits but still producing the minor subunit (designated FimD$^+$). The ability of the two fimbrial mutants to colonize the nasopharynx, trachea, and lungs was compared with those of the wild type parental strain and a filamentous hemagglutinin (FHA) mutant. Of the three mutants studied, the FimD$^-$ mutant showed the greatest defect, colonizing less well in the nasopharynx, trachea, and lungs. The most pronounced defect in colonizing ability of the three mutants was observed in the trachea. However, the colonizing defect of the FHA and FimD$^+$ mutants in the trachea was observed only during the first 3 days of infection. After 10 days, the colonization level was nearly restored to wild-type levels. The FHA and FimD$^+$ mutants showed a slight colonization defect in the nasopharynx but no defect in the lungs. A maltose binding protein-FimD fusion protein and a peptide derived from FimD were able to bind to heparin, a member of a class of sulfated sugars which are ubiquitous in the respiratory tract. Recently it was shown (W. L. W. Hazenbos, C. A. W. Geuijen, B. M. van den Berg, F. R. Mooi, and R. van Furth, J. Infect. Dis. 171:924–929, 1995) that FimD also binds to the integrin VLA-5, and our results suggest that the binding of *B. pertussis* to these two molecules plays an important role in colonization of the respiratory tract of the mouse.**

Bordetella pertussis is the causative agent of whooping cough, a highly contagious and serious infection of the respiratory tract. Adherence of *B. pertussis* to the respiratory tract is the first and essential step in pathogenesis, and two filamentous proteins play an important role in this process, the filamentous hemagglutinin (FHA) and fimbriae. FHA is a multifunctional molecule for which three ligands have been described, all of which are present in the respiratory tract: sulfated sugars, lactosylceramides, and the CR3 receptor (3, 14, 15, 24, 35). Sulfated sugars are ubiquitous in the respiratory tract and are found in mucus secretions, in the extracellular matrix (ECM), and on epithelial cells (38), while lactosylceramides are found in the ciliary membranes of the respiratory epithelium and on alveolar macrophages (35). The CR3 receptor is present on macrophages, and the binding of *B. pertussis* to CR3 results in uptake of the bacterium into the phagocyte, where it is able to survive for an extended period (6, 30). Like FHA, *B. pertussis* fimbriae are also filamentous; however, they belong to a different class of proteins. *B. pertussis* is able to produce two serologically distinct fimbriae, the serotype 2 and 3 fimbriae, which are composed of the major subunits Fim2 and Fim3, respectively (21, 27). In addition to these major subunits, the fimbriae contain a single minor fimbrial subunit species, designated FimD (41). Expression of the major fimbrial subunits, Fim2 and Fim3, is regulated by small insertions and deletions in the C-rich promoter region, resulting in fimbrial phase variation (39). Two ligands have been defined for *B. pertussis* fimbriae: the major fimbrial subunits bind to sulfated sugars, whereas the minor fimbrial subunit shows affinity for VLA-5, an integrin which is found on macrophages (7, 8). Hazenbos et al. (8, 9) have shown that binding of *B. pertussis* to monocytes via VLA-5 activates CR3, the receptor for FHA. Thus, in vivo FHA and fimbriae may cooperate to invade alveolar macrophages. The major fimbrial subunit genes are scattered on the chromosome (34), but FimD is part of a gene cluster involved in fimbrial and FHA biosynthesis (Fig. 1) (40–43). Thus, the *fha* and *fim* genes are linked and intermingled, and their products cooperate in the invasion of macrophages. Strains with well-defined mutations in the major fimbrial subunit genes, *fim2* and *fim3*, have been constructed and analyzed in animal models (19, 20). The discovery of the minor fimbrial subunit raised the possibility that Fim2-Fim3 mutants may still express FimD at their cell surfaces and therefore do not represent true fimbrial mutants. In this work we constructed a true fimbrial mutant, devoid of both major and minor fimbrial subunits. The ability of this mutant to colonize mice was compared to those of the wild-type strain, a Fim2-Fim3 mutant, and an FHA mutant. Furthermore, we show that, like the major fimbrial subunits, FimD is able to bind the sulfated sugar heparin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. Conditions for growth have been described previously (18). Antibiotics were used in the following concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; gentamicin, 50 μg/ml; and streptomycin, 300 μg/ml.

DNA techniques. Unless otherwise stated, standard methods were used for plasmid and chromosomal DNA isolation, restriction enzyme digestion, and agarose gel electrophoresis (29). Labeling and detection of DNA was performed with a nonradioactive DNA labeling and detection kit (Boehringer Mannheim GmbH).

* Corresponding author. Mailing address: Research Laboratory for Infectious Diseases, National Institute of Public Health and Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands. Phone: 3130-2743091. Fax: 3130-2744449. E-mail: FR.Mooi@rivm.nl.

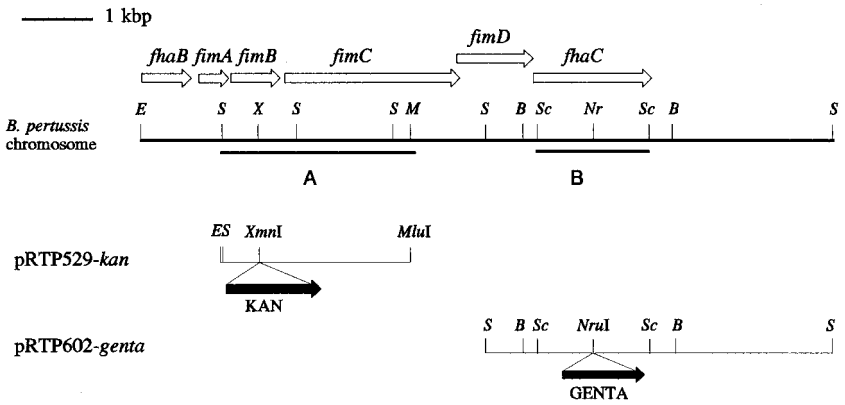

FIG. 1. Physical and genetic map of the *B. pertussis* chromosomal region containing *fha* and *fim* genes. The position and direction of transcription of genes is indicated by open arrows. Inserts contained in plasmids pRTP529-*kan* and pRTP602-*genta* used to construct fimbrial and FHA mutants are indicated. Probes A and B were used to check whether the required insertion in the chromosome was obtained. Only restriction sites relevant to this study are shown. *E*, *Eco*RI; *S*, *Sal*I; *X*, *Xmn*I; *M*, *Mlu*I; *B*, *Bcl*I; *Sc*, *Sca*I; *Nr*, *Nru*I.

Construction of FHA and Fim mutants. The *fimB* mutant was constructed by introducing a kanamycin resistance (KAN) cassette into the *fimB* gene by allelic exchange with pRTP529-*kan*. Plasmid pRTP529-*kan* contains a *B. pertussis* 2.8-kbp *Sal*I-*Mlu*I DNA fragment, in which *fimB* was inactivated by insertion of a KAN cassette into the *Xmn*I site of *fimB*. To minimize the effects of this insertion on the expression of *fhaC*, the KAN cassette was inserted in the same transcriptional orientation as the *fimB* gene (Fig. 1). Plasmid pRTP529-*kan* was used to transfer the KAN cassette to the chromosomal *fimB* gene of strain B536 by allelic exchange with strain SM10 as donor as described previously (32). The correct insertion of the KAN cassette was checked by Southern blotting of *Sal*I-digested chromosomal DNA from the parental strain and a number of exconjugants, using probe A (Fig. 1). In the parental strain and the exconjugants bands of 1.3 and 2.7 kbp, respectively, were found to hybridize with probe A. The difference in size between these bands is in accordance with insertion of the 1.4-kbp KAN cassette. One *fimB*::*kan* mutant, designated B316, was used for further studies.

To inactivate the *fhaC* gene, a gentamicin resistance (GENTA) gene was inserted into the chromosomal *fhaC* gene by gene replacement with pRTP602-*genta* essentially as described for *fimB*. Plasmid pRTP602-*genta* contains a 4.4-kbp *Sal*I fragment in which the *fhaC* gene is inactivated by insertion of a GENTA gene into the *Nru*I site located in *fhaC* (Fig. 1) in the same transcriptional orientation as *fhaC*. The GENTA cassette was derived from pSS1129 (33). The correct insertion of the GENTA cassette was checked by Southern blotting of *Bcl*I-digested chromosomal DNA from the parental strain and a number of exconjugants, using probe B (Fig. 1). In the parental strain and the exconjugants bands of 2.3 and 3.5 kbp, respectively, were found to hybridize with probe B. The difference in size between these bands is in accordance with insertion of the 1.2-kbp GENTA cassette. One *fhaC*::*genta* mutant, designated B317, was used for further studies.

Immunological techniques. Total-cell lysates containing approximately 5×10^7 bacteria were analyzed by immunoblotting, essentially as described by Geuijen et al. (7), using monoclonal antibodies (23). A sodium dodecyl sulfate–10% polyacrylamide gel was used for the separation of FHA, and a 15% gel was used for the separation of fimbrial subunits. The amount of FHA and fimbriae produced by the strains was quantitated by a whole-cell enzyme-linked immunosorbent assay (ELISA) essentially as described by Willems et al. (43). Briefly, 5×10^7 bacteria, suspended in 0.1 M sodium carbonate buffer (pH 9.6), were used to coat microtiter plates (Corning Costar) and the water was evaporated overnight. The plates were washed with running tap water and blocked by incubation with PBSTB (phosphate-buffered saline [PBS] [pH 7.0], 1% bovine serum albumin [BSA] [Boseral PM; Organon Teknika], 0.05% Tween 20) for 1 h at room temperature. The amount of FHA and fimbriae was determined by incubation with serial dilutions of the monoclonal antibodies 31E2, 228B7, and 36G3, raised against FHA, serotype 2 fimbriae, and lipopolysaccharide, respectively (23). Subsequently, the plates were washed and incubated with sheep anti-mouse peroxidase conjugate. Finally, the wells were washed and incubated with a peroxidase substrate (0.4 mM 3,3′,5,5′-tetramethylbenzidine [Sigma Chemical Co., St. Louis, Mo.]–0.09% H_2O_2 in 110 mM sodium acetate buffer [pH 5.5]), and after stopping the reaction by adding 3 M H_2SO_4, we determined the optical density at 450 nm (OD_{450}). Incubation with anti-lipopolysaccharide monoclonal antibody was performed in order to determine whether there were differences in coating efficiencies between *B. pertussis* strains. No differences were observed.

Immunoelectron microscopy of bacteria. Bacteria were grown for 2 days at 35°C in Verwey medium (37), washed two times in PBS, suspended to an OD_{600} of 1.0, and fixed for 30 min in 2% paraformaldehyde with 0.5% glutaraldehyde. For immunogold labeling, Formvar- and carbon-coated nickel grids were placed on the bacterial suspension. Subsequently, the grids were incubated in 0.05 M glycine–PBS for 30 min at room temperature, followed by incubation with a 1:100 dilution of a mixture of different anti-FimD monoclonal antibodies for 30 min and then with gold (6-nm diameter)-conjugated goat anti-mouse immunoglobulin (diluted 1:100; EY Labs, Inc., San Mateo, Calif.) for 60 min. After the final washing, the bacteria were fixed with 1% glutaraldehyde in PBS. The grids were negatively stained with 1% ammonium molybdate and analyzed in a Philips TEM400 electron microscope.

Mouse experiments. BALB/c/Rivm mice about 3 weeks old were lightly anesthetized with ether, and a drop of 5 μl of the inoculum, containing 10^5 *B. pertussis* cells, was placed on top of each nostril and allowed to be inhaled by the animal. For the determination of bacterial colonization, the mice were killed by intraperitoneal injection of an overdose of a barbiturate (pentobarbital sodium [Nembutal]; Sanofi/Algin), after which the respiratory tract was excised. The lungs were cut into small pieces and homogenized in 500 μl of Verwey medium on a vortex with glass beads. The tracheas were excised and homogenized in 500 μl of Verwey medium, and the nasopharynxes were sampled by introducing 500 μl of Verwey medium from inside the animal and collecting the first eight drops exiting from the nostrils. The suspensions were plated in 10-fold serial dilutions on Bordet Gengou plates supplemented with streptomycin (30 μg/ml) and colony-forming units were counted after 3 to 4 days of incubation at 35°C.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype or genotype	Source or reference
B. pertussis		
B536	$Fim2^+$ $Fim3^-$	25
B52	Derived from B536; *fim2*::*sacI* *fim3*::*kan* $Fim2^-$ $Fim3^-$ $FimD^+$	20
B316	Derived from B536; *fimB*::*kan* $Fim2^-$ $Fim3^-$ $FimD^-$	This study
B317	Derived from B536; *fhaC*::*genta* FHA^-	This study
E. coli		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96* φ80 *lacZΔM15*	Bio-Rad, Gaithersburg, Md.
SM10	*thi thr leu supIII* RP4-Tc::Mu Kan	31
Plasmids		
pRTP1	Amp *rpsL oriT cos*	32
pSS1129	Amp Genta *rpsL oriT cos*	33
pRTP529-*kan*	pRTP1 with *fimB*::*kan*	40
pRTP602	pRTP1 with 3′ end of *fimD* and *fhaC*	This study
pRTP602-*genta*	Derived from pRTP602 with *fhaC*::*genta*	This study

TABLE 2. Adhesins expressed by the parental strain and mutant derivatives

Strain	Genotype	Phenotype[a]			
		Fim2	Fim3	FimD	FHA
B536	Parental	+	−	+	+
B52 (FimD$^+$)	*fim2*::*sacI fim3*::*kan*	−	−	+[b]	+
B316 (FimD$^-$)	*fimB*::*kan*	−	−	−	+[c]
B317 (FHA$^-$)	*fhaC*::*genta*	+	−	+	−

[a] Phenotypes were examined by immunoblotting (Fim2, Fim3, FimD, and FHA) and ELISA (Fim2 and FHA). +, present; −, absent.
[b] Reduced compared to strain B536.
[c] Seventy-five percent of the level expressed by B536.

Adherence of biotinylated heparin to proteins and peptides. *B. pertussis* fimbriae, FimD-maltose binding protein (MBP) (7), or MBP was used to coat microtiter plates overnight at room temperature at a concentration of 5 μg/ml in PBS. FimD-derived synthetic peptides (see below) were coated at a concentration of 20 μg/ml for 2 h at 37°C. After being coated, plates were washed four times with PBS containing 0.05% Tween 20 (PBST) in a 96-plate washer (SLT, 96PW; Proton-Wilson) and blocked by incubation with heat-treated PBSB (1% BSA in PBS) for 1 h at room temperature. Heat treatment of BSA consisted of incubation at 56°C for 60 min and filtration of the solution through a 0.22-μm-pore-size filter (22). Plates were incubated overnight with biotinylated heparin (Sigma) at room temperature, washed four times with PBST, and incubated with a streptavidin-peroxidase conjugate (1:1,000 dilution; Amersham) for 2 h at 37°C. Finally, the plates were washed four times with PBST and 100 μl of a peroxidase substrate (0.4 mM 3,3′,5,5′-tetramethylbenzidine [Sigma]–0.09% H_2O_2 in 110 mM sodium acetate buffer [pH 5.5]) was added to each well. After 5 min the reaction was stopped by adding 50 μl of 3 M H_2SO_4 and the OD_{450} was determined.

Peptides. Peptides A-I-K-V-G-P-L-K-R-P-R-K-L-V-L and V-Q-L-I-N-G-K-T-Q-Q-P-V-K-L-G were derived from FimD residues 183 to 197 and 313 to 327, respectively. Peptides were synthesized on a Biolynx 4170 automated synthesizer (Pharmacia/LKB, Uppsala, Sweden) according to the method of Van der Ley et al. (36).

Statistical methods. Statistical significance was determined by use of Student's *t* test after logarithmic transformation of the data, using STATA software (Computing Resource Center, Santa Monica, Calif.).

RESULTS

Construction and characterization of fimbrial and FHA mutants. Immunoblotting indicated that FimD was still produced by strain B52 (Table 2). Furthermore, functional assays indicated that it was surface exposed in strain B52 (7, 8). To confirm the latter finding, we performed immunoelectron microscopy with monoclonal antibodies directed against FimD. When the wild-type strain was investigated, the gold label was found to be associated with filamentous structures, most likely representing the serotype 2 fimbriae (Fig. 2). The gold particles were concentrated in particular regions of the fimbrial rod. Thus, FimD is not distributed along the whole length of the fimbrial shaft but is possibly located at the fimbrial tip. When strain B52 was analyzed, no gold particles were found associated with filamentous structures or with the bacterial cell surface.

To construct a *B. pertussis* strain completely devoid of both major and minor fimbrial subunits, the fimbrial chaperon gene, *fimB*, was inactivated by insertional inactivation, resulting in strain B316. It has been shown that fimbrial chaperon mutants accumulate fimbrial subunits in the periplasmic space, where they are degraded (2, 17, 40). An FHA mutant (strain B317) was constructed by insertional inactivation of *fhaC*, which is required for export of the FHA molecule (12, 43). The production of FHA, Fim2, Fim3, and FimD by the constructed strains was examined by immunoblotting or ELISA and compared to the parental strain (Table 2). The mutants revealed the expected phenotypes; however, it was found that strain B52 produced less FimD than the parental strain whereas strain B316 expressed 75% of the amount of FHA produced by the parental strain. The latter phenomenon may be due to a polar effect of the insertion in *fimB* on the expression of *fhaC*, since both genes are probably part of a single operon (Fig. 1) (43). Finally, the effect of the mutations on growth rate in vitro was determined, and no significant differences were found between the wild-type strain and its mutant derivatives (results not shown). The two fimbrial mutants used in this study, B316 and B52, will be referred to as the FimD$^-$ and FimD$^+$ mutants, respectively.

Analysis of fimbrial and FHA mutants in a mouse model. Mice were infected intranasally with the wild-type strain or one of the mutant strains, and the amount of bacteria in the nasopharynx, trachea, and lungs was determined directly after infection and 1, 3, and 10 days postinfection (Fig. 3). In the nasopharynx, only small differences were observed between the wild-type strain and the FHA and FimD$^+$ mutants, both of which colonized slightly less well on days 1 and 3 compared to the wild-type strain. The difference between the wild-type strain and the FimD$^-$ mutant was more pronounced. Statistically significant lower amounts of this mutant were recovered in all three experiments on days 1, 3, and 10.

In the trachea, all three mutants were less well able to colonize than the wild-type strain during the first 3 days. However, on day 10, the colonization of the trachea by the FimD$^+$ and FHA mutants was nearly restored to wild-type levels. In contrast, the FimD$^-$ mutant was not recovered on day 10. Furthermore, this strain was recovered in smaller amounts on days 0, 1, and 3 than the other strains.

In the lungs, the FHA and FimD$^+$ mutants seemed to colonize slightly better than the wild-type strain. However, the differences were generally not statistically significant. The FimD$^-$ mutant was consistently recovered in smaller amounts than the other strains. Statistically significant differences between the FimD$^-$ mutant and the wild-type strain were observed on days 3 and 10.

FimD binds to heparin. The analysis of fimbrial mutants in the mouse model indicated that FimD plays an important role in the colonization of the mouse respiratory tract, and this raised the question of which receptor FimD binds to in these tissues. Previously (7) we presented evidence that strains expressing FimD were better able to adhere to immobilized sulfated sugars, like heparin and heparan sulfate, than strains devoid of FimD, suggesting that FimD may bind sulfated sugars. We were indeed able to detect binding of heparin to a MBP-FimD fusion protein in a dose-dependent manner, while no binding of heparin to MBP was observed (Fig. 4A). It has been shown that proteins binding to heparin contain stretches of basic amino acids (5). To identify a heparin binding region within FimD, the amino acid sequence of FimD was screened for the region which possessed the highest concentration of basic amino acids. A peptide derived from this region, comprising residues 183 to 197, bound biotinylated heparin (Fig. 4B). No heparin binding was observed with a control peptide derived from another region of FimD (i.e., amino acids 313 to 327) (Fig. 4B).

DISCUSSION

In this study we provide evidence that the minor subunit of *B. pertussis* fimbriae, FimD, is a major factor in the colonization of the mouse respiratory tract. Immunoelectron microscopy indicated that FimD is not distributed along the fimbrial shaft but is found concentrated in particular regions, possibly at the fimbrial tip. Minor fimbrial subunits produced by other bacteria are often found at the tips of the fimbriae, as in the

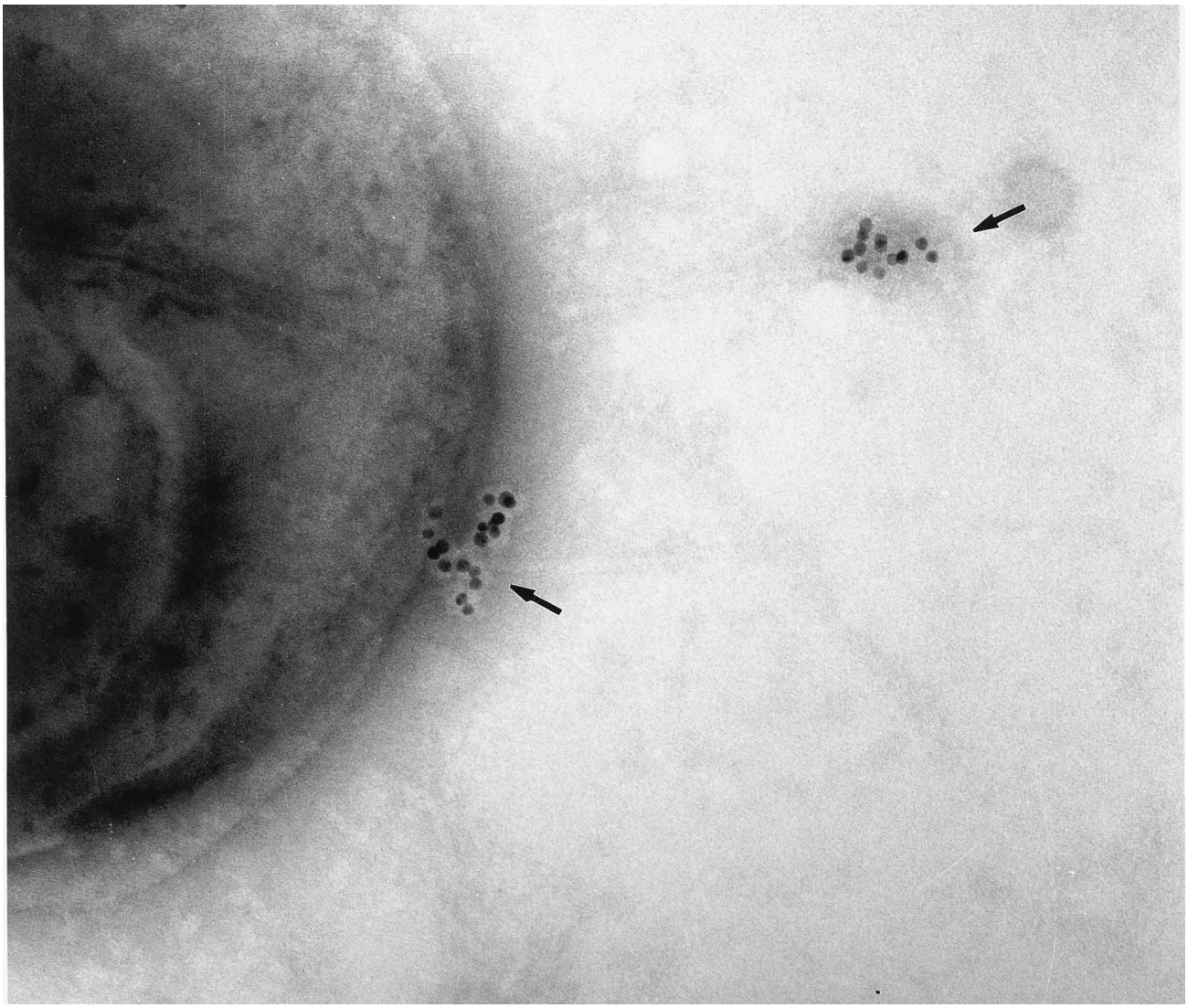

FIG. 2. Transmission electron microscopy of a wild-type *B. pertussis* bacterium immunolabeled with a mixture of monoclonal antibodies which recognize FimD. The arrows indicate gold label. Note the presence of two labeled fimbriae, one of which is extending from the cell surface while the other is folded back on itself. Magnification, ×352,000.

case of Pap and type 4 fimbriae of *Escherichia coli* and *Neisseria gonorrhoeae*, respectively (11, 28). However, in type I, S, F9, and 987P fimbriae of *E. coli* the minor subunits are localized at the tip and occur periodically along the length of the fimbrial shaft (1, 4, 16, 26).

Immunoblotting indicated that FimD is still produced by a mutant, designated B52, in which both major fimbrial subunit genes have been inactivated (Table 2). However, we were not able to determine by immunoelectron microscopy whether FimD is surface exposed on strain B52. The amount of FimD produced by strain B52, which is less than that of the wild-type strain, may be below the detection level of immunoelectron microscopy. Also, FimD in strain B52 may be less accessible to antibodies than when it is assembled into the fimbrial rod, or it may be present in a conformation which is not recognized by the monoclonal antibodies used. Functional studies implied that FimD is exposed on the surface of strain B52 (7, 8). For example, *B. pertussis* is able to bind to monocytes through FimD, and this binding activity is still expressed by strain B52 but not by a mutant devoid of FimD (8). Significantly, binding of strain B52 ($FimD^+$), but not of a $FimD^-$ strain, was inhibited by purified fimbriae and MBP-FimD, clearly implying that strain B52 binds to monocytes via FimD. Likewise, B52, but not a strain devoid of FimD, is able to adhere to sulfated sugars (7).

The availability of a pair of mutant strains differing only in the expression of the minor subunit allowed us to assess the contribution of FimD to the pathogenesis of pertussis in a mouse model. Compared to the other strains tested, the $FimD^-$ mutant showed the lowest level of colonization at all three sites sampled (Fig. 3). The most pronounced defect in colonizing ability of the $FimD^-$ mutant was observed in the trachea. In the nasopharynx, the colonization defect of the $FimD^-$ mutant was less dramatic, although the strain was consistently recovered in smaller numbers than the wild-type strain. In the lungs, a difference in colonizing ability between the wild-type strain and the $FimD^-$ mutant was most pronounced late in infection (day 10). The FHA and $FimD^+$

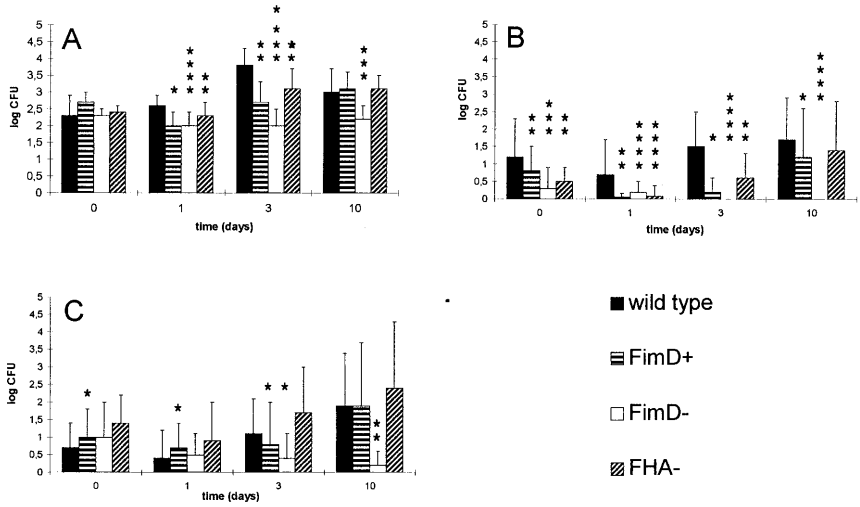

FIG. 3. Growth of the *B. pertussis* parental strain and its mutant derivatives in the nasopharynx (A), trachea (B), and lungs (C) of mice. Each bar represents the geometric mean of log colony-forming units from 10 mice. The error bars indicate standard deviations. Experiments were performed three (with strain FimD$^+$) or four times, and representative results are shown. Asterisks indicate the number of experiments which showed statistically significant differences in colonization between mutant and wild-type strains. Wild type, strain B536; FimD$^+$, mutant strain B52; FimD$^-$, mutant strain B316; FHA$^-$, mutant strain B317.

mutants showed their largest defect in colonizing ability in the trachea during the first 3 days. On day 10, however, the colonization level was nearly restored to wild-type levels. A recolonization of the trachea by *B. pertussis* mutants has been described previously (10) and may be due to tissue destruction, resulting in a less efficient clearance by ciliary movement or in the unmasking of novel receptors. A slight colonization defect of the FHA and FimD$^+$ mutants was observed during the first 3 days in the nasopharynx. In agreement with previous studies (10, 19, 20), the FHA and FimD$^+$ mutants showed no colonization defect in the lungs.

The FHA production of the FimD$^-$ strain was reduced to 75% of wild-type levels, thus raising the possibility that the colonization behavior of the FimD$^-$ strain was partly due to smaller amounts of FHA produced. However, the FHA mutant, which was completely devoid of FHA, was far less affected in its ability to colonize the mouse respiratory tract than the FimD$^-$ mutant. Thus, it is reasonable to conclude that the colonization defect of the FimD$^-$ mutant was caused primarily by the absence of FimD.

The important role FimD plays in colonization of the mouse respiratory tract raises the question of which host receptors are involved. Previous work has identified the integrin VLA-5 as a ligand for FimD (9). Here we show that an MBP-FimD fusion protein, but not MBP, binds to the sulfated sugar heparin (Fig. 4A), thus identifying a second ligand for FimD. Earlier studies with *B. pertussis* fimbrial mutants also suggested that FimD was able to bind to heparin (7). Heparin binding sites are characterized by a high density of basic amino acids, and a peptide derived from FimD which fulfilled these criteria was indeed able to bind heparin (Fig. 4B). It should be noted that, although we have shown that FimD binds to sulfated sugars and VLA-5 (9), we have not formally demonstrated that the colonization defect of the FimD$^-$ mutant is due to the inability to interact with these molecules. Apart from FimD, two other *B. pertussis* adhesins, FHA and the major fimbrial subunits, also confer the ability to bind to sulfated sugars (7, 14, 15), and cooperation between two or more of these adhesins may be required for stable binding, as has been observed in vitro (7). Sulfated sugars occur in distinct compositions, which show different tissue distributions (38), and it is conceivable that FHA and fimbriae have different fine specificities for sulfated sugars, allowing tropism to those sites where optimal receptors for both *B. pertussis* adhesins are expressed.

Adhesins are not only involved in passive adherence but may also contribute to persistence in the host by interfering with

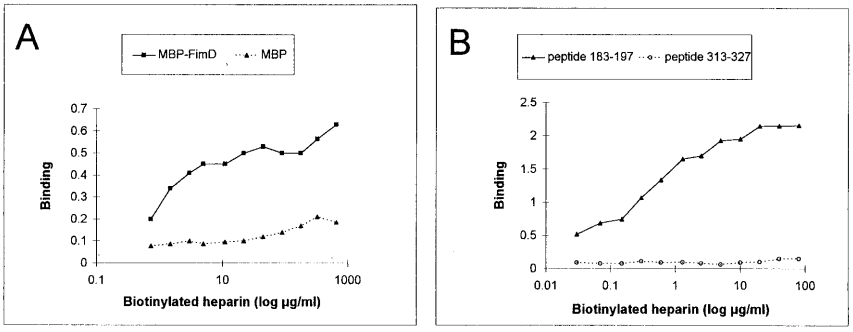

FIG. 4. Adherence of heparin to the FimD-MBP fusion protein (A) or FimD peptides (B). Proteins or peptides were adsorbed onto microtiter plates and incubated with biotinylated heparin. Adherent heparin was detected with a streptavidin-conjugated peroxidase. The assay was carried out at least three times, and a representative result is shown.

natural host-ligand interactions, and the interaction of FimD with VLA-5 may serve such a function. Passive adherence is not expected to be important in the lung alveoli, where mechanical clearance of bacteria by mucociliary movement does not occur. Indeed, of the three adhesin mutants, only the FimD$^-$ mutant showed a defect in its ability to persist in the lung. In the lung alveoli, the first line of defense is constituted of alveolar macrophages, and since FimD binds to macrophages through the VLA-5 receptor it may affect macrophage function. A role for FimD in invasion of macrophages has been proposed by Hazenbos et al. (9). However, this process is dependent on the presence of FHA (9), and since the FHA mutant was not affected in its ability to persist in the mouse lung, invasion of alveolar macrophages probably does not contribute to persistence of *B. pertussis* in the mouse colonization model. Nevertheless, FimD may hamper macrophage function in other ways than invasion. Binding of FimD to monocytes activates a protein tyrosine kinase and may therefore affect intracellular signaling (9).

It is interesting that FimD shows the same binding specificities as fibronectin, which has distinct binding domains for VLA-5 and sulfated sugars. Fibronectin is a widely distributed cell-adhesive glycoprotein present in the ECM and in other body fluids which displays several functions, including tissue response to injury and cell migration. Interaction of fibronectin with VLA-5 leads to the assembly of soluble fibronectin into the ECM during tissue injury (13). Competition of FimD with fibronectin for receptor sites can therefore disrupt fundamental host functions. Further in vivo and in vitro studies may clarify the role of FimD in pathogenesis.

ACKNOWLEDGMENTS

We are grateful to Frank Bekius and Hans van Kooten for technical assistance, Marina Burger and J. S. Teppema for electron microscopy, and Jan Poolman and Betsy Kuipers for monoclonal antibodies.

C.G. was supported by the Dutch Organization for Scientific Research (NWO) grant no. 901-14-135.

REFERENCES

1. **Abraham, S. N., J. D. Goguen, D. Sun, P. Klemm, and E. H. Beachey.** 1987. Identification of two ancillary subunits of *Escherichia coli* type I fimbriae by using antibodies against synthetic oligopeptides of *fim* gene products. J. Bacteriol. **169:**5530–5536.
2. **Bakker, D., E. E. M. Vader, B. Roosendaal, F. R. Mooi, B. Oudega, and F. K. de Graaf.** 1991. Structure and function of periplasmic chaperon-like proteins involved in the biosynthesis of K88 and K99 fimbriae in enterotoxigenic *Escherichia coli*. Mol. Microbiol. **5:**875–886.
3. **Brennan, M. J., J. H. Hannah, and E. Leininger.** 1991. Adhesion of *Bordetella pertussis* to sulfatides and to the GalNAcβ4Gal sequence found in glycosphingolipids. J. Biol. Chem. **266:**18827–18831.
4. **Cao, J., A. S. Khan, M. E. Bayer, and D. M. Schifferli.** 1995. Ordered translocation of 987P fimbrial subunits through the outer membrane of *Escherichia coli*. J. Bacteriol. **177:**3704–3713.
5. **Cardin, A. D., and H. J. R. Weintraub.** 1989. Molecular modeling of protein-glycosaminoglycan interactions. Arteriosclerosis **9:**21–32.
6. **Friedman, R. I., K. Nordensson, L. Wilson, E. T. Aakporiaye, and D. E. Yocum.** 1992. Uptake and intracellular survival of *Bordetella pertussis* in human macrophages. Infect. Immun. **60:**4578–4585.
7. **Geuijen, C. A. W., R. J. L. Willems, and F. R. Mooi.** 1996. The major fimbrial subunit of *Bordetella pertussis* binds to sulfated sugars. Infect. Immun. **64:**2657–2665.
8. **Hazenbos, W. L. W., C. A. W. Geuijen, B. M. van den Berg, F. R. Mooi, and R. van Furth.** 1995. Binding of *Bordetella pertussis* to human monocytes: role of FimD. J. Infect. Dis. **171:**924–929.
9. **Hazenbos, W. L. W., B. M. van den Berg, C. A. W. Geuijen, F. R. Mooi, and R. van Furth.** 1995. Binding of FimD on *Bordetella pertussis* to very late antigen-5 on monocytes activates complement receptor type 3 via protein tyrosine kinase. J. Immunol. **155:**3972–3978.
10. **Kimura, A., K. T. Mountzouros, D. A. Relman, S. Falkow, and J. L. Cowell.** 1990. *Bordetella pertussis* filamentous hemagglutinin: evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. Infect. Immun. **60:**2380–2385.
11. **Lindberg, F., B. Lund, L. Johansson, and S. Normark.** 1987. Localization of the receptor-binding protein adhesin at the tip of the bacterial pilus. Nature **328:**84–86.
12. **Locht, C., P. Bertin, F. D. Menozzi, and G. Renauld.** 1993. The filamentous haemagglutinin, a multifaceted adhesin produced by virulent *Bordetella* spp. Mol. Microbiol. **9:**653–660.
13. **McDonald, J. A.** 1988. Extracellular matrix assembly. Annu. Rev. Cell Biol. **4:**183–207.
14. **Menozzi, F. D., C. Gantiez, and C. Locht.** 1991. Interaction of *Bordetella pertussis* filamentous haemagglutinin with heparin. FEMS Microbiol. Lett. **78:**59–64.
15. **Menozzi, F. D., R. Mutombo, G. Renauld, C. Gantiez, J. H. Hannah, E. Leininger, M. J. Brennan, and C. Locht.** 1994. Heparin-inhibitable lectin activity of the filamentous haemagglutinin adhesin of *Bordetella pertussis*. Infect. Immun. **62:**769–778.
16. **Moch, T., H. Hoschützky, J. Hacker, K.-D. Kröncke, and K. Jann.** 1987. Isolation and characterization of the α-sialyl-β-2,3-galactosyl specific adhesin from fimbriated *Escherichia coli*. Proc. Natl. Acad. Sci. USA **84:**3462–3466.
17. **Mooi, F. R., A. Wijfjes, and F. K. De Graaf.** 1983. Identification and characterization of precursors in the biosynthesis of the K88ab fimbriae of *Escherichia coli*. J. Bacteriol. **154:**41–49.
18. **Mooi, F. R., H. G. J. van der Heide, A. R. ter Avest, K. G. Welinder, I. Livey, B. A. M. van der Zeist, and W. Gaastra.** 1987. Characterization of fimbrial subunits from *Bordetella* species. Microb. Pathog. **12:**473–484.
19. **Mooi, F. R., H. G. J. van der Heide, H. C. Walvoort, H. Brunings, W. H. Jansen, and P. A. M. Guinee.** 1991. The analysis of *Bordetella pertussis* fimbrial mutants in a rabbit model, p. 69–76. *In* E. Z. Ron and S. Rottem (ed.), Microbial surface components and toxins in relation to pathogenesis. Plenum Publishing Corp., New York, N.Y.
20. **Mooi, F. R., W. H. Jansen, H. Brunings, H. Gielen, H. G. J. van der Heide, H. C. Walvoort, and P. A. M. Guinee.** 1992. Construction and analysis of *Bordetella pertussis* mutants defective in the production of fimbriae. Microb. Pathog. **12:**127–135.
21. **Mooi, F. R.** 1994. Genes for the filamentous hemagglutinin and fimbriae of *Bordetella pertussis*: colocation, coregulation, and cooperation?, p. 145–155. *In* V. L. Miller, J. B. Kaper, D. A. Portnoy, and R. R. Isberg (ed.), Molecular genetics of bacterial pathogenesis. ASM Press, Washington, D.C.
22. **Ortegia-Barria, E., and M. E. A. Pereira.** 1991. A novel T. cruzi heparin-binding protein promotes fibroblast adhesion and penetration of engineered bacteria and trypanosomes into mammalian cells. Cell **67:**411–421.
23. **Poolman, J. T., B. Kuipers, M. L. Vogel, H. J. Hamstra, and J. Nagel.** 1990. Description of a hybridoma bank towards *Bordetella pertussis* toxin and surface antigens. Microb. Pathog. **8:**377–382.
24. **Relman, D., E. Tuomanen, S. Falkow, D. T. Golenbock, K. Saukkonen, and D. W. Wright.** 1990. Recognition of a bacterial adhesin by an integrin: macrophage CR3 (I_MJ_2, CD11b-/CD18) binds filamentous hemagglutinin of *Bordetella pertussis*. Cell **61:**1375–1382.
25. **Relman, D. A., M. Domenghini, E. Tuomanen, R. Rappuoli, and S. Falkow.** 1989. Filamentous hemagglutinin of *Bordetella pertussis*: nucleotide sequence and crucial role in adherence. Proc. Natl. Acad. Sci. USA **86:**2637–2641.
26. **Riegman, N., H. Hoschützky, I. van Die, W. Hoekstra, K. Jann, and H. Bergmans.** 1990. Immunocytochemical analysis of P-fimbrial structure: localization of minor subunits and the influence of the minor subunit FsoE on the biogenesis of the adhesin. Mol. Microbiol. **4:**1193–1198.
27. **Robinson, A., L. A. E. Ashworth, and L. I. Irons.** 1989. Serotyping *Bordetella pertussis* strains. Vaccine **7:**491–494.
28. **Rudel, T., I. Scheurerpflug, and T. F. Meyer.** 1995. Neisseria PilC protein identified as type-4 pilus tip-located adhesin. Nature **373:**357–359.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Saukkonen, K., C. Cabellos, M. Burroughs, S. Prasad, and E. Tuomanen.** 1991. Integrin-mediated localization of *Bordetella pertussis* within macrophages: role in pulmonary colonization. J. Exp. Med. **173:**1143–1149.
31. **Simon, R., U. Priefer, and A. A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
32. **Stibitz, S., W. Black, and S. Falkow.** 1986. The construction of a cloning vector designed for gene replacement in *Bordetella pertussis*. Gene **50:**133–140.
33. **Stibitz, S., and M.-S. Yang.** 1991. Subcellular localization and immunological detection of proteins encoded by the *vir* locus of *Bordetella pertussis*. J. Bacteriol. **173:**4288–4296.
34. **Stibitz, S., and T. L. Garletts.** 1992. Derivation of a physical map of the chromosome of *Bordetella pertussis* Tohama I. J. Bacteriol. **174:**7770–7777.
35. **Tuomanen, E., H. Towbin, G. Rosenfelder, D. Braun, G. Larson, G. Hansson, and R. Hill.** 1988. Receptor analogs and monoclonal antibodies that inhibit adherence of *Bordetella pertussis* to human ciliated respiratory epithelial cells. J. Exp. Med. **168:**267–277.
36. **Van der Ley, P., J. E. Heckels, M. Virji, P. Hoogerhout, and J. T. Poolman.** 1991. Topology of outer membrane porins in pathogenic *Neisseria* spp. Infect. Immun. **59:**2963–2971.

37. **Verwey, W. F., E. H. Thiele, D. N. Sage, and L. T. Suchardt.** 1949. A simplified liquid culture medium for the growth of *Haemophilus pertussis*. J. Bacteriol. **58:**127–134.
38. **Wight, T. N., D. K. Heingard, and V. C. Hascall.** 1991. Proteoglycans structure and function, p. 45–78. *In* E. G. Hay (ed.), Cell biology of extracellular matrix, 2nd ed. Plenum Publishing Corp., New York, N.Y.
39. **Willems, R. J. L., H. G. J. van der Heide, A. R. ter Avest, and F. R. Mooi.** 1990. Fimbrial phase variation in *Bordetella pertussis*: a novel mechanism for transcriptional regulation. EMBO J. **9:**2803–2809.
40. **Willems, R. J. L., H. van der Heide, and F. R. Mooi.** 1992. Characterization of a *Bordetella pertussis* fimbrial gene cluster which is located directly downstream of the filamentous hemagglutinin gene. Mol. Microbiol. **6:**2661–2671.
41. **Willems, R. J. L., C. Geuijen, H. G. J. van der Heide, M. Matheson, A. Robinson, R. Ebberink, J. Theelen, and F. R. Mooi.** 1993. Isolation of a putative fimbrial adhesin from *Bordetella pertussis* and the identification of its gene. Mol. Microbiol. **9:**623–634.
42. **Willems, R. J. L.** 1993. Genetic and functional studies on *Bordetella pertussis* fimbriae. Ph.D. dissertation. Utrecht University, Utrecht, The Netherlands.
43. **Willems, R. J. L., C. Geuijen, H. G. J. van der Heide, G. Renauld, P. Bertin, W. M. R. van den Akker, C. Locht, and F. R. Mooi.** 1994. Mutational analysis of the *Bordetella pertussis fim/fha* gene cluster: identification of a gene with sequence similarities to haemolysin accessory genes involved in export of FHA. Mol. Microbiol. **11:**337–347.

Editor: P. J. Sansonetti