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The Filamentous Hemagglutinin of *Bordetella parapertussis* Is the Major Adhesin in the Phase-Dependent Interaction with NCI-H292 Human Lung Epithelial Cells

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***Bordetella parapertussis* is a Gram-negative bacterium which colonizes the human respiratory tract and can cause whooping cough or pertussis. This pathogen is subject to phase variation and expresses a series of virulence factors exclusively in the Bvg$^+$ phase. Here, it is demonstrated for the first time that only the Bvg$^+$ phase of *B. parapertussis* adheres to and invades the human lung epithelial cell line NCI-H292. A *B. parapertussis* mutant defective in expression of the Bvg$^+$-regulated filamentous hemagglutinin (FHA) showed reduced binding (77% reduction) to NCI-H292 cells, as did a FHA mutant of the related *Bordetella pertussis* (85% reduction). In contrast to *B. pertussis*, binding of *B. parapertussis* to NCI-H292 cells was not inhibited by heparin, suggesting differences in the FHA adhesin and its host-cell receptor between these two species. Thorough understanding of the mechanism of action of the *B. parapertussis* virulence factors, such as FHA, is of particular interest in the development of novel strategies of pertussis vaccination.**

The Gram-negative bacteria *Bordetella parapertussis* and *Bordetella pertussis* are etiological agents of pertussis or whooping cough in humans (1). The clinical symptoms of *B. parapertussis* infection are generally less severe in comparison to those caused by *B. pertussis* (2,3). *B. parapertussis* infections are not always recognized, and recent data have shown that *B. parapertussis* infections are more common than previously thought (4). The pathogens differ in several important features including chromosome size (the *B. parapertussis* genome is about 1 megabase larger (5)), lipopolysaccharide expression (6,7) and pertussis toxin (Ptx) expression (8). Ptx is only produced by *B. pertussis* and plays an important role in the infection process, whereas *B. parapertussis* does not express this virulence factor. Furthermore, it is of importance to note that *B. parapertussis* and *B. pertussis* are immunologically different (9).

The expression of most virulence determinants of *B. pertussis*, including Ptx, filamentous hemagglutinin (FHA), fimbriae and adenylate cyclase toxin, is coordinately regulated by the two-component response regulatory system BvgAS (10). Environmental factors such as temperature and sulfate-anion concentration can modulate this system, and mutations in *BvgAS* can inactivate the locus (phase variation) and consequently result in avirulence of the bacterium (11, 12). *B. parapertussis* has a similar BvgAS system (13) which has also been implicated in the expression of several virulence components. In contrast to *B. pertussis*, the BvgAS of *B. parapertussis* is involved in lipopolysaccharide expression (14).

Bacterial adhesion to (ciliated) epithelial cells of the respiratory tract is a crucial step in the pathogenesis of *Bordetella* infections (15). In order to gain a better understanding of the disease process, it is of importance to know the bacterial and host cell factors involved in this process. This understanding can help to explain the host and tissue tropism of the pathogens as well as provide strategies for prevention. The present study was directed toward identifying *B. parapertussis* components playing a role in this binding process. Here, it is demonstrated for the first time that *B. parapertussis* binds in a Bvg-dependent manner to NCI-H292 human lung epithelial cells. Furthermore, the *B. parapertussis* component homologous to *B. pertussis* FHA is identified as a major adhesin in the interaction with these lung epithelial cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *B. parapertussis* strain B24 is a human clinical isolate with a Bvg$^+$ phenotype (16) and strain B24.3 is a spontaneous phase variant derived from B24 (14). *B. pertussis* strain BP338 is a nalidixic acid derivative of Tohama I with a Bvg$^+$ phenotype (17). BP338ery is a spontaneous

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phase variant of BP338 (7). The construction of *fhaC* mutants in *B. parapertussis* (B24FHAC) and in *B. pertussis* (BP536FHAC) is described below. The bordetellae were cultured on Bordet-Gengou (BG) agar (Difco Laboratories) supplemented with 15% (v/v) defibrinated sheep blood. *E. coli* was grown on Luria-Bertani agar (18). Plates were incubated in a humidified atmosphere containing 5% (v/v) CO_2 at 37°C. Antibiotics were used at the following concentrations: gentamycin 50 μg ml^{-1}; nalidixic acid 30 μg ml^{-1}; streptomycin 300 μg ml^{-1}; kanamycin 50 μg ml^{-1}.

Culture of epithelial cell lines. The human epithelial cell line NCI-H292 (American Type Culture Collection: CRL 1848) originates from a pulmonary mucoepidermoid carcinoma and the HeLa cell line is derived from a human cervix epitheloid carcinoma (American Type Culture Collection: CCL 2). The cell lines were maintained in RPMI 1640 tissue culture medium with 2 mM L-glutamine (Gibco BRL) and supplemented with 5% (v/v) fetal bovine serum. The cell lines were cultured in humidified air containing 5% (v/v) CO_2 at 37°C.

Infection experiments. To study quantitatively the interaction between bacteria and epithelial cells, the following assay was used. Bacteria grown on BG agar were collected with a sterile cotton swab and suspended in phosphate-buffered saline containing Ca^{2+} and Mg^{2+} cations (PBSCM: 2.68 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8.0 mM Na_2HPO_4, 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.2). An appropriate amount of this bacterial suspension was added to the culture medium of a 90% to 100% confluent monolayer of an epithelial cell culture in 24-well tissue culture plates (Costar), at a multiplicity of infection (MOI) of 200:1 (bacteria to epithelial cells). After 4 hours of incubation under the same conditions as used for the cell culture, the cell layer was washed three times with PBSCM to remove non-associated bacteria. To release the intracellular bacteria from the epithelial cells, the cell layer was incubated with 200 μl of a saponin (Serva) solution (1 μg ml^{-1} in culture medium) for 15 min at 37°C. After repeated pipetting and vortexing, the suspension was plated out in serial dilutions on BG agar to determine the number of colony forming units (cfu).

In order to quantitate bacterial invasion, a polymyxin B-based protection assay was used. The bordetellae are sensitive to the concentration of polymyxin B used, but this antibiotic does not penetrate the eukaryotic cell wall and thus leaves the intracellular bacteria unaffected (19). Four hours after the start of infection, non-adherent bacteria were washed away and extracellular bacteria were killed by incubating in culture medium containing 100 μg ml^{-1} polymyxin B for 2 hours. After washing twice in PBSCM, the intracellular bacteria were released by saponin treatment and the number of cfu was determined as described above. In order to measure long-term intracellular survival, the infected cell lines were then cultured further after the 2 hour polymyxin B treatment in the presence of polymyxin B (10 μg ml^{-1}). This treatment should eliminate any bordetellae which might have escaped from the host cell. The subsequent procedure was identical to that used in the invasion assay.

The study of bacterial adhesion with light microscopy after crystal violet staining was carried out as described previously (20).

Transmission electron microscopy. NCI-H292 cells were grown on Transwell polycarbonate membrane filters (#3421, Costar) to 90% confluency and infected with *B. parapertussis* strain B24 at a MOI of 500:1. After allowing the infection to proceed for 5 hours, filters were washed three times with PBSCM and the cells were fixed with 2% glutaraldehyde in 0.2 M HEPES (pH 7.4) at 4°C. For transmission electron microscopy, glutaraldehyde-fixed filters were immersed in 1% Osmium tetraoxide (OsO_4; Science Services, Munich, Germany) in PBS for 1 hour and, after dehydration through a graded series of ethanol, ultra-thin sections were stained using 1% aqueous uranyl acetate and lead citrate. Stained filters were cut out, laid their apical sides uppermost and then embedded in Epon. Samples were cut with a Diatome diamond knife (Reicher-Jung, Vienna, Austria) using an ultramicrotome (LKB, Stockholm, Sweden) and were viewed using a Philips CM10 electron microscope.

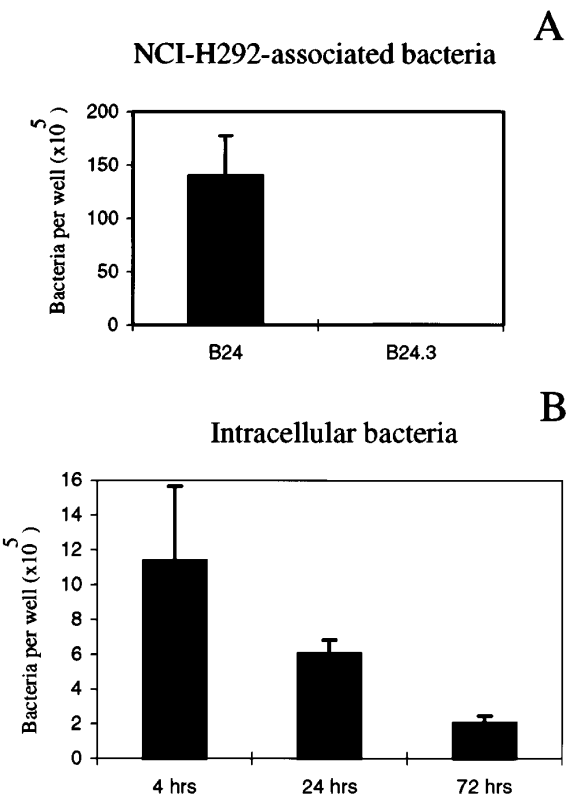

FIG. 1. Phase-dependent interaction of *B. parapertussis* with NCI-H292 human lung epithelial cells. A. The number of Bvg$^+$ phase (B24), and Bvg$^-$ phase (B24.3) *B. parapertussis* bacteria associated with NCI-H292 cells was determined 4 hours after the start of infection. B. The number of viable intracellular Bvg$^+$ phase *B. parapertussis* (B24) bacteria was determined in a polymyxin B-based protection assay at the time points indicated. No intracellular Bvg$^-$ phase (B24.3) bacteria were detected (data not shown). The data represent mean values ± SD of two independent experiments each carried out in duplicate.

Construction and analysis of Bordetella mutants deficient in FHA expression. In *B. parapertussis*, the *B. pertussis fhaC* gene homologue was inactivated by integration of plasmid pRIP633 (21) into the chromosome of a streptomycin- and nalidixic acid-resistant derivative of strain B24 (B24SmNal). The plasmid was introduced into B24SmNal via bacterial conjugation as described elsewhere (14). FHA expression was analyzed by Western blotting of whole-cell lysates separated in a 8% SDS-PAGE gel (7), using the *B. pertussis* FHA monoclonal antibody 31E2 (22).

A kanamycin cassette was introduced into the *fhaC* gene of BP536, a streptomycin and nalidixic acid-resistant derivative of the *B. pertussis* Tohama I strain (23), as described previously for the construction of a *fhaC*::kan mutant in the Wellcome strain of *B. pertussis* (21). As expected, the *fhaC*::kan mutant (designated BP536FHAC) expressed minimal amounts of FHA (data not shown).

RESULTS

Bvg$^+$ Phase but Not the Bvg$^-$ Phase of B. parapertussis Adheres to and Invades NCI-H292 Lung Epithelial Cells

The interaction of the Bvg$^+$ and the Bvg$^-$ phase of *B. parapertussis* with NCI-H292 human lung epithelial

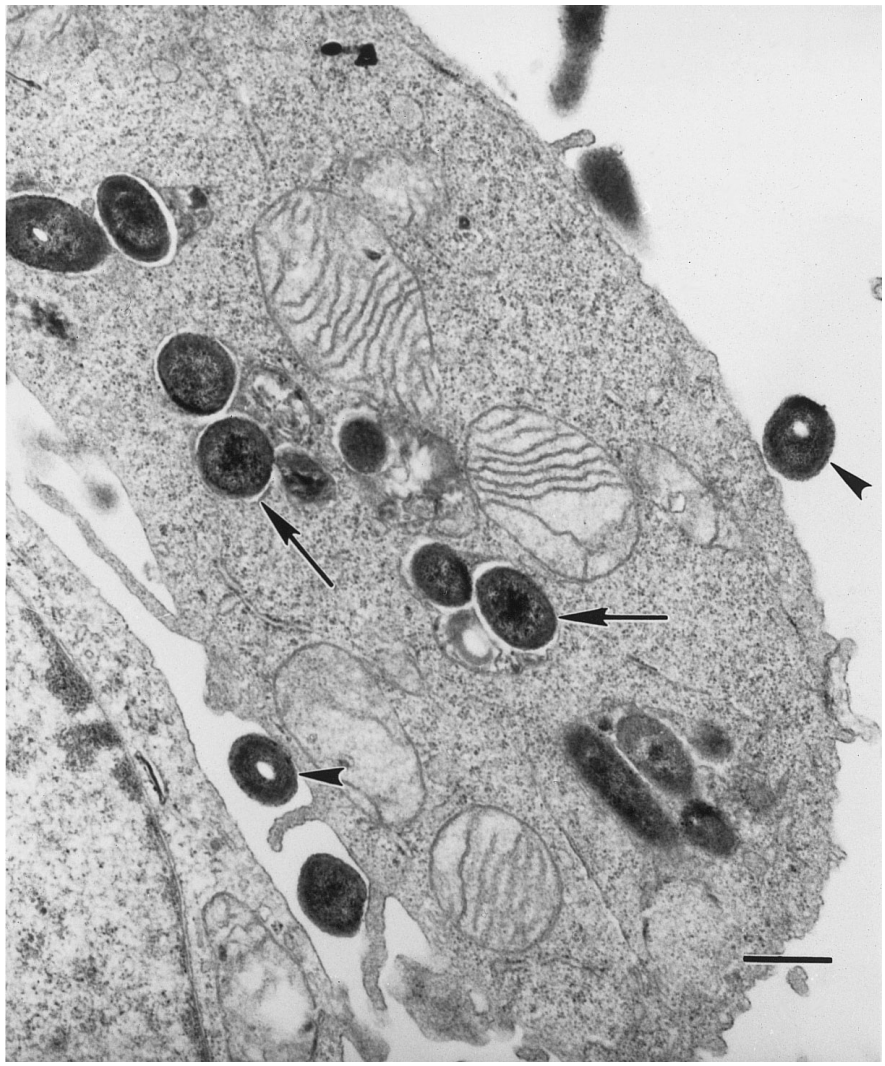

FIG. 2. Transmission electron micrograph of NCI-H292 lung epithelial cells 5 hours after infection with *B. parapertussis* strain B24. Arrows indicate intracellular bacteria bound by endocytic vacuoles, and arrowheads indicate adherent bacteria. Bar represents 0.6 μm.

cells was compared. Four hours after the start of infection, the Bvg^+ phase (B24) was strongly adherent whereas the number of Bvg^- phase variant (B24.3) adherent bacteria was less than 2% the level seen with strain B24 (Fig. 1A). Light-microscopic examination of crystal-violet stained infected NCI-H292 cells confirmed this result (data not shown).

To investigate whether the *B. parapertussis* Bvg^+ phase is invasive for NCI-H292 cells, transmission electron microscopic studies were performed. After 5 hours of infection many bacteria were observed intracellularly (Fig. 2). Most of these bacteria were singular and surrounded by tight vacuoles, and occasionally clusters of up to 8 intracellular bacteria were observed. A polymyxin B-based protection assay was used to determine the number of intracellular viable bacteria. 8 $\pm$ 3% the total number of NCI-H292 associated bacteria appeared to be intracellular after 4 hours. This number of intracellular bacteria decreased gradually over a period of 3 days to 18 $\pm$ 4 % of the number which were intracellular at 4 hours (Fig. 1B). During this long-term survival experiment, the NCI-H292 cells seemed to be in good condition and formed a confluent monolayer. The Bvg^- phase of *B. parapertussis* (strain B24.3) did not invade the NCI-H292 cells. These observations suggest that Bvg^+-regulated factors of *B. parapertussis* are essential in the invasion process.

Construction of a B. parapertussis Mutant Deficient in FHA Expression

B. parapertussis expresses a component showing similarities with the FHA of *B. pertussis* (24). Since *B. pertussis* FHA has been reported to function as an adhesin in the bacterial interaction with epithelial cells (25, 26), we investigated whether FHA of *B. para-*

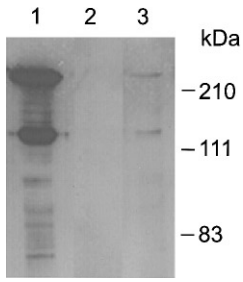

FIG. 3. Western blot showing FHA expression in *B. parapertussis*. The blot displays full-length FHA with a molecular mass of about 220 kDa, and several degradation products. Lane 1, strain B24 (Bvg^+); Lane 2, strain B24.3 (Bvg^-), and Lane 3, strain B24FHAC (*fhaC*::pRIP633 mutant). Molecular-mass markers are indicated on the right.

pertussis has a similar function. To create a *B. parapertussis* mutant deficient in FHA expression, the *B. pertussis fhaC* homologue in *B. parapertussis* strain B24 was inactivated by insertion of the pRIP633 plasmid (a suicide plasmid containing part of the *B. pertussis fhaC* gene (21)) via homologous recombination. *fhaC* encodes a protein that is thought to function as a chaperone, and has an essential function in the transport of FHA (21, 27). Analogous to the *B. pertussis* system, the *B. parapertussis fhaC* mutant (designated B24FHAC) expresses minimal amounts of FHA (Fig. 3). In addition to the full-length 220 kDa FHA, several degradation products of FHA were detected. Furthermore, FHA expression was absent in the phase variant of *B. parapertussis* (B24.3), suggesting that its expression is controlled by the BvgAS regulatory system.

B. parapertussis FHA Deficient Mutant Is Impaired in Binding to NCI-H292 Cells

The interaction of the *B. parapertussis* mutant deficient in FHA expression with NCI-H292 cells was studied. As shown in Figure 4A, the FHA mutant showed a reduction in binding capacity of 77 ± 3 % in comparison with its parental strain B24. Heparin, a glycosaminoglycan, has been reported to inhibit the binding of *B. pertussis* to HeLa cells (28, 29). In the presence of 50 or 100 μg ml^{-1} heparin, no reduction in the binding of *B. parapertussis* to NCI-H292 cells was detected (Fig. 4A). In contrast, the binding of *B. pertussis* was strongly inhibited by the presence of 50 μg ml^{-1} heparin (Fig. 4B). A clear reduction (85%) in adhesion was observed with a *B. pertussis* mutant deficient in FHA expression (strain BP536FHAC). A *B. pertussis* phase variant (BP338ery) bound to NCI-H292 cells at a level less than 1% of the level shown for BP338.

It has been demonstrated that a *B. pertussis* FHA anti-serum recognises the FHA of *B. parapertussis* but that this serum was unable to block adhesion to HeLa cells (30). To investigate a possible role of *B. parapertussis* FHA in the bacterial adhesion to HeLa cells, the

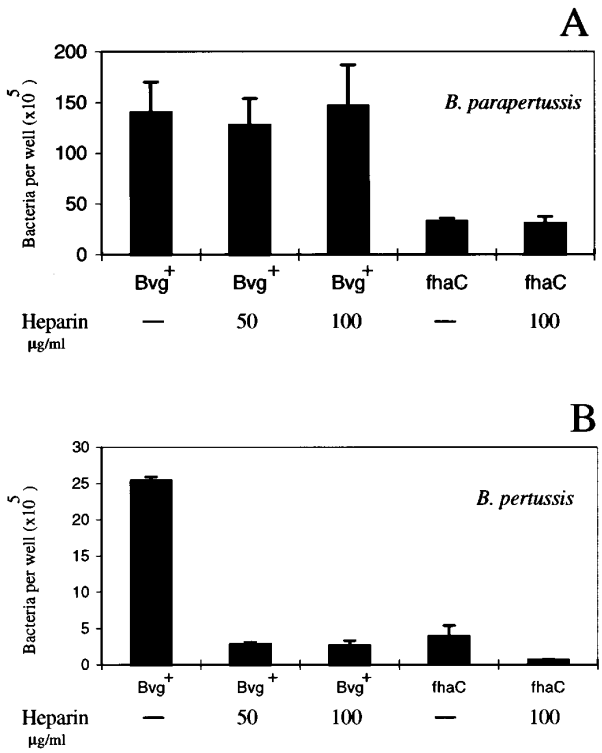

FIG. 4. Association of Bvg^+ phase, and FHA deficient mutants of *B. parapertussis* (A), and *B. pertussis* (B) with NCI-H292 lung epithelial cells. The presence of heparin, and its concentrations used in the experiments are indicated. *B. parapertussis* strains: Bvg^+, B24; fhaC, B24FHAC. *B. pertussis* strains: Bvg^+, BP338; fhaC, BP536FHAC. A representative result of two to three independent experiments performed in duplicate is shown.

FHA deficient strain B24FHAC was tested for its ability to adhere to this cell line. It was found that the adherence of this *B. parapertussis* FHA mutant to HeLa cells was reduced to 71 ± 5 % of the level shown for strain B24 (Fig. 5). The *B. parapertussis* phase

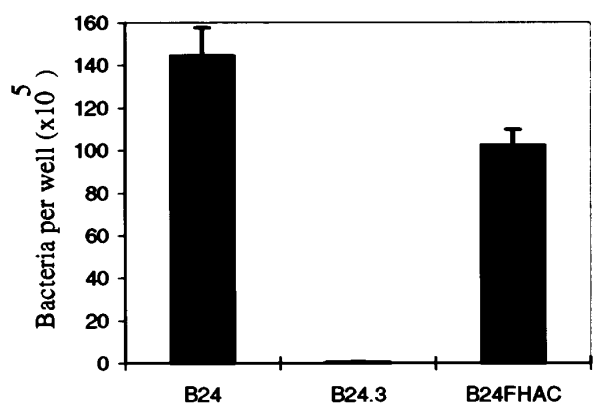

FIG. 5. Association of *B. parapertussis* strains B24 (Bvg^+ phase), B24.3 ((Bvg^- phase), and B24FHAC (FHA-deficient mutant) with HeLa cells. Data represent mean values ± SD of three experiments performed in duplicate.

variant (B24.3) was strongly impaired in its ability to interact with HeLa cells (less than 0.3% of B24).

DISCUSSION

In the present study, it is shown that Bvg^+-regulated components of the respiratory pathogen *B. parapertussis* are essential for adherence to NCI-H292 human lung epithelial cells. It is further demonstrated that interaction of *B. pertussis* with this cell line is similarly phase dependent. In contrast, *Bordetella bronchiseptica*, another related pathogen of the respiratory tract, binds independently of its Bvg-status to NCI-H292 cells (20).

Since FHA of *B. pertussis* has been suggested to function as a major adhesin in the interaction with some epithelial cell lines (23, 31), we investigated whether the homologue of FHA in *B. parapertussis* plays a role in the interaction with the NCI-H292 cell line. Both in case of *B. parapertussis* and *B. pertussis*, FHA deficient mutants showed a clear reduction in binding to NCI-H292 cells (77% and 85%, respectively). This indicates that the FHA of both bacterial species functions as important adhesins in this system. The failure to inhibit *B. parapertussis* binding to HeLa cells with a *B. pertussis* FHA antiserum suggested that *B. parapertussis* FHA was not required for binding to this cell type (30). In the present study, however, binding of a FHA deficient *B. parapertussis* mutant to HeLa cells was shown to be reduced by 23 $\pm$ 3 % in comparison with its parental strain, indicating a minor role of FHA in the adhesion of *B. parapertussis* to HeLa cells.

Interestingly, it was observed that heparin was able to inhibit the binding of *B. pertussis* to NCI-H292 cells, whereas no effect of this substance was found in case of *B. parapertussis* (Fig. 4). Heparin-inhibitable adhesion of *B. pertussis* is reported for WiDr cells, hamster trachea cells (28) as well as HeLa cells (29). This effect is can be explained by a direct interaction of heparin with *B. pertussis* FHA (32) and thereby blocking bacterial adherence. The failure of heparin to block adhesion of *B. parapertussis* to NCI-H292 cells suggests that heparin does not bind to *B. parapertussis* FHA or, alternatively, that a possible binding of heparin to FHA does not interfere with the region of FHA required for binding to the host cell. Heparan-sulfate glycosaminoglycans, in addition to glycophingolipids, are widely distributed over human epithelial cells and both supposedly function as receptors for *B. pertussis* FHA (28, 33, 34). The above differences between *B. parapertussis* and *B. pertussis* FHA might indicate that these two species make use of different receptors on NCI-H292 cells.

Binding of *B. parapertussis* to NCI-H292 and HeLa cells was not reduced to the level of the Bvg^- phase variant, even after abolishment of FHA expression, implying that other Bvg-regulated components are involved in the adhesion process as well. Based on studies with *B. pertussis*, Bvg-regulated components that have corresponding components in *B. parapertussis* and are associated with host-cell binding include pertactin (35) and fimbriae (26, 37). It remains to be determined whether these factors function as additional adhesins for *B. parapertussis*.

The identification of *B. parapertussis* FHA as an important adhesin and the observed differences with the *B. pertussis* homologue, is of particular interest regarding the replacement of the *B. pertussis* whole-cell vaccines by acellular vaccines in some countries. The acellular pertussis vaccines consist solely of *B. pertussis* components that elicit a weaker protection against *B. parapertussis* in comparison with the *B. pertussis* whole cell vaccine in respiratory mouse models (9, 38). The use of *B. parapertussis* FHA in the acellular pertussis vaccine should therefore be considered.

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