Production of Nontypeable *Haemophilus influenzae* HtrA by Recombinant *Bordetella pertussis* with the Use of Filamentous Hemagglutinin as a Carrier

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*Bordetella pertussis*, the etiologic agent of whooping cough, is a highly infectious human pathogen capable of inducing mucosal and systemic immune responses upon a single intranasal administration. In an attenuated, pertussis toxin (PTX)-deficient recombinant form, it may therefore constitute an efficient bacterial vector that is particularly well adapted for the delivery of heterologous antigens to the respiratory mucosa. Filamentous hemagglutinin (FHA) has been used as a carrier to present foreign antigens at the bacterial surface, thereby inducing local, systemic, and protective immune responses to these antigens in mice. Both full-length and truncated (Fha44) forms of FHA have been used for antigen presentation. To investigate the effect of the carrier (FHA or Fha44) on antibody responses to passenger antigens, we genetically fused the HtrA protein of nontypeable *Haemophilus influenzae* to either FHA form. The fha-htrA and Fha44-gene-htrA hybrids were expressed as single copies inserted into the chromosome of PTX-deficient *B. pertussis*. Both chimeras were secreted into the culture supernatants of the recombinant strains and were recognized by anti-FHA and anti-HtrA antibodies. Intranasal infection with the strain producing the FHA-HtrA hybrid led to significantly higher anti-HtrA and anti-FHA antibody titers than those obtained in mice infected with the Fha44-HtrA-producing strain. Interestingly, the *B. pertussis* strain producing the Fha44-HtrA chimera colonized the mouse lungs more efficiently than the parental, Fha44-producing strain and gave rise to higher anti-FHA antibody titers than those induced by the parental strain.

The mucosal delivery of vaccines has several advantages over systemic delivery. Consequently, the development of efficient mucosal vaccine delivery systems, including live attenuated or commensal bacterial vaccines, has attracted much attention over the last decades. However, most efforts have so far been devoted to the oral route, although the oral delivery of vaccine formulations requires large quantities of antigen and repeated administrations to be effective. The nasal route may represent an interesting alternative (23), as it avoids the competition with commensal microorganisms present in the digestive tract.

*Bordetella pertussis*, the etiologic agent of whooping cough, has recently been used as a live vector to deliver foreign antigens to the respiratory mucosa in laboratory models (29). As a highly contagious agent, it colonizes the human respiratory tract very efficiently and induces both local and systemic immune responses. The deletion of the genes encoding pertussis toxin (PTX) of *B. pertussis* has recently been used as a live vector to deliver foreign antigens, has attracted much interest, and repeated administrations to be effective. The nasal route may represent an interesting alternative (23), as it avoids the competition with commensal microorganisms present in the digestive tract.

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body responses against both Fha44 and TbpB after i.n. administration (7).

For this study, we engineered *B. pertussis* strains to produce HtrA from nontypeable *Haemophilus influenzae* (NTHI) fused to either Fha44 or full-length FHA in order to investigate the effect of the carrier protein on the immunogenicity of the passenger antigen. HtrA was used as a model antigen because it is a naturally secreted monomeric protein produced by NTHI, in contrast to previously used antigens, which were either cytosolic or part of multimeric structures in their natural hosts. NTHI is a major cause of otitis media in young children and of lower respiratory tract infections in adults, with recurrent episodes of the disease (19, 32), and i.n. immunization has been demonstrated to be an effective means of reducing the colonization of NTHI in the nasal tract (20, 21). HtrA is a stress response protein with serine protease activity that belongs to the \( \sigma^D \)-dependent family of heat shock proteins (6). It is well conserved among NTHI strains and has been shown to elicit partial protection in infant rat and chinchilla models (5, 26), which makes this protein an attractive candidate for a subunit vaccine. HtrA has been identified as a virulence factor in *Legionella pneumophila*, *Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica*, and *Brucella abortus* (9, 17, 22, 33). However, the role of HtrA in the pathogenesis of NTHI remains to be determined.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The *B. pertussis* strains used for this study are listed in Table 1. They were all derived from a PTX-deficient Tohama I derivative named BPRA (4) and were grown on Bordet-Gengou agar (Difco, Detroit, Mich.) supplemented with 1% glycerol, 20% defibrinated sheep blood, and 100 \( \mu \)g/ml streptomycin (Sigma Chemical Co., St Louis, Mo.) at 37°C for 72 h. Liquid cultures of *B. pertussis* were incubated as described previously (27) in Stainer-Scholte medium containing 1 \( \mu \)liter heptakis(2,6-di-o-methyl) \( \beta \)-cyclodextrin (Sigma). For cell adherence assays, exponentially growing *B. pertussis* was inoculated at an optical density at 600 nm of 0.15 in 2.5 ml of Stainer-Scholte medium supplemented with 65 \( \mu \)M/I (1\%)-S)methionine plus 1\%-S)lysine (NEN, Boston, Mass.) and then was grown for 24 h at 37°C. The bacteria were then washed three times with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 (Gibco, Grand Island, N.Y.) at the desired density.

**B. pertussis mutant constructs.** B. pertussis BPSA85, BPSA199, and BPSA167 were obtained by allelic exchange as described by Stibitz (40), using the pQomp200pL18 (34) derivatives pAS28 (to produce the Fha44-HtrA hybrid), pAS86 (to produce the FHA-HtrA hybrid), and pAS65 (to produce the Fha44-HtrA* hybrid), a protein in which the HtrA RGD sequence was changed to RAD (9, 17, 22, 33). How-

**Antigens.** FHA was purified from *B. pertussis* BPRA by heparin-Sepharose chromatography, as described previously (28). Purified FHA was kindly provided by S. Loosmore (Aventis-Pasteur, Toronto, Canada).

**Cells and growth conditions.** The human pulmonary epithelial cell line A549 (ATCC CL-185) was cultured in RPMI medium containing sodium penicillin G (1,000 U/ml), streptomycin (50 \( \mu \)g/ml) (Gibco), 2 mM l-glutamine (Gibco), and 10\%-inactivated fetal calf serum (Gibco), using uncoupled tissue culture flasks and 24-well plates. The murine alveolar macrophage cell line MH-S (ATCC CRL-2019) was propagated in uncoupled tissue culture flasks and 24-well plates in the same RPMI-based medium as that described above, supplemented with 1.5 g of sodium bicarbonate/liter, 4.5 g of glucose/liter, 10 mM HEPES, 1 mM sodium pyruvate, and 50 \( \mu \)M \( \beta \)-mercaptoethanol. Cells were detached mechanically by scraping.

**Cell adherence assay.** Cells (2 \( \times \) 10^6 per well) were cultured for 2 days in 24-well plates. The cells were then washed once with RPMI medium before the addition of 4 \( \times \) 10^5 \( ^{35} \)S-labeled bacteria per well and incubation for 1 h on 30 min at 37°C in 5\% CO\( _2 \). After three washes with RPMI medium to remove nonadherent bacteria, the cells were lysed with 0.5% sodium dodecyl sulfate. The labeling of the whole-cell lysates was quantified by liquid scintillation count-

**Intranasal infection.** *B. pertussis* grown on BG agar was suspended in sterile PBS and adjusted to a concentration of approximately 2.5 \( \times \) 10^5 CFU/ml for colonization studies or 2.5 \( \times \) 10^6 CFU/ml for immunogenicity studies. Infections were performed by the i.n. route as described before (1), using 9-week-old BALBc mice (Iffa Credo, L’Arbresle, France). For colonization studies, the lungs were aseptically removed at the indicated time points and homogenized in PBS. Serial dilutions of individual lung homogenates were plated onto BG agar, and the numbers of CFU were determined after 3 to 4 days of incubation at 37°C. Four to 8 mice per time point and per group of mice were assessed. All animal studies were carried out under the guidelines of the Institut Pasteur de Lille animal study board.

**Antibody detection.** The levels of antibodies to HtrA and FHA were measured with enzyme-linked immunosorbent assays (ELISAs). Microwell plates (Maxi- sorp; Nunc) were coated with 50 \( \mu \)l of 0.05 M carbonate buffer (pH 9.6; Sigma) containing 5 \( \mu \)g/ml purified antigen. After blocking of the samples with PBS containing 0.1% Tween and 1% bovine serum albumin, 50 \( \mu \)l of serum was added to each well of the plate. The plates were incubated for 2 h at 37°C, and goat anti-mouse total IgG-horseradish peroxidase or biotinylated goat anti-IgA with a conjugate (Amersham, Les Ulis, France) was added at 1/4,000 or 1/6,000 dilution, respectively. For anti-IgA detection, peroxidase-conjugated streptavidin (Amersham) was added at a 1/4,000 dilution. The ELISAs were developed by using ABTS ([2,2’-azinobis(3-ethylbenzthiazolsulfonic acid); Boehringer Mannheim, Germany] according to the manufacturer’s specifications. The results of the insertion of the 3.815-bp Sall fragment from pAS64 into Sall-digested pQomp200pL18. pAS64 was obtained by inserting the BamHI fragment from pAS60 into BamHI-digested pAS3. pAS60 resulted from the insertion of a PCR product into PCRRItope. This PCR product was generated by the use of pBR7H91A and the oligonucleotides 5’-CCGGGATCCACTTTGCCAAA-3’ and 5’-GCGAGATTATTGGCACTAATAAATAGAAATTAC3’.

## TABLE 1. *B. pertussis* strains used for this study

<table>
<thead>
<tr>
<th>B. pertussis strain</th>
<th>Relevant phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPRA</td>
<td>FHA* -ΔPTX</td>
<td>4</td>
</tr>
<tr>
<td>BPSA85</td>
<td>Fha44-HtrA* -ΔPTX</td>
<td>This study</td>
</tr>
<tr>
<td>BPSA87</td>
<td>Fha44* -ΔPTX</td>
<td>2</td>
</tr>
<tr>
<td>BPSA167</td>
<td>Fha44-HtrA** -ΔPTX</td>
<td>This study</td>
</tr>
<tr>
<td>BPSA199</td>
<td>FHA-HtrA* -ΔPTX</td>
<td>This study</td>
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are expressed as the highest dilutions of sera yielding an absorbency at 405 nm three times above the control values.

**Statistical analysis.** The results were analyzed by use of the unpaired Student \( t \) test. Differences were considered significant at \( P \) values of \( < 0.05 \).

**RESULTS**

**Production of chimeric Fha44-HtrA and FHA-HtrA by recombinant *B. pertussis*.** The 46-kDa HtrAH91A antigen was fused either to full-length FHA by use of the unique BglII site of the *fhaB* gene or to the C terminus of Fha44, a truncated form corresponding to the N-terminal 80-kDa portion of mature FHA (35). Since HtrA expresses proteolytic activity (26), we chose the enzymatically inactive mutant form HtrAH91A to avoid potential interference of the proteolytic activity with its immunogenicity. The chimeric proteins were named FHA-HtrA and Fha44-HtrA, respectively. The constructs were introduced by double homologous recombination into the chromosomal *fhaB* locus of PTX-deficient *B. pertussis* BPRA.

The production of the chimeric proteins by the recombinant *B. pertussis* strains was analyzed by immunoblotting of culture supernatants. As shown in Fig. 1, polypeptides of the expected sizes (approximately 130 kDa for Fha44-HtrA and 270 kDa for FHA-HtrA) were detected by using an anti-HtrA, anti-Fha44, or anti-FHA antibody, indicating that these chimeric proteins contained both HtrA and Fha44-FHA epitopes. Several smaller polypeptides were also detected, most likely corresponding to proteolytic breakdown products of the chimeras. In addition, the chimeras were also detected at the bacterial cell surface by immunoblotting (data not shown). These results indicate that both FHA and Fha44 are competent carrier proteins allowing *B. pertussis* to secrete the 46-kDa HtrA protein. However, the supernatant from the culture of FHA-HtrA-producing *B. pertussis* was concentrated 10-fold in order to detect the FHA-HtrA chimera, whereas the Fha44-HtrA hybrid protein was readily detected in unconcentrated supernatants. In contrast, there were approximately equal amounts of cell-associated Fha44-HtrA and FHA-HtrA (not shown), indicating that Fha44 is a more efficient carrier protein than full-length FHA with respect to the level of secretion.

**Lung colonization by recombinant *B. pertussis*.** To study the ability of the recombinant *B. pertussis* strains to colonize the respiratory tract, we infected mice i.n. with BPSA85 or BPSA199 and compared the colonization profiles to those of the parental strains. The colonization profile observed with the FHA-HtrA-producing strain BPSA199 was very similar to that observed with the corresponding parental strain, BPRA, indicating that the fusion of HtrA to full-length FHA does not alter the ability of *B. pertussis* to colonize the lungs of mice (Fig. 2A). In contrast, the recombinant strain BPSA85, which produced Fha44-HtrA, colonized mice significantly more efficiently than the parental Fha44-producing strain BPSA87 (Fig. 2B). This result indicates that the fusion of HtrA to the C terminus of Fha44 overcomes the previously described defect in colonization of a *B. pertussis* strain producing Fha44 instead of FHA in a PTX-deficient background (2). Adherence studies using the human pulmonary epithelial cell line A549 (Fig. 3A) or the murine

![FIG. 1. Production and secretion of Fha44-HtrA and FHA-HtrA by *B. pertussis*. Twenty microliters of unconcentrated culture supernatant from *B. pertussis* BPSA85 (A and B), BPSA87 (A and B), or BPRA (C and D) or 20 \( \mu \)l of 10-fold-concentrated culture supernatant from BPSA199 (C and D) was analyzed by immunoblotting using anti-HtrA (A and C), anti-Fha44 (B), or anti-FHA (D) antibodies. The sizes of the molecular mass markers, expressed in kDa, are given on the right.](http://iai.asm.org/)

![FIG. 2. Lung colonization by *B. pertussis*. BALB/c mice were infected i.n. with approximately 5 \( \times \) 10^5 CFU of *B. pertussis* BPRA (black bars in panel A), BPSA199 (white bars in panel A), BPSA87 (black bars in panel B), BPSA85 (white bars in panels B and C), or BPSA167 (black bars in panel C). At the indicated times after challenge, the mice were sacrificed, and the viable bacteria present in the lungs were counted. Four mice from each group were analyzed per time point. The dashed lines in panels B and C represent the limit of bacterial counts. * P < 0.05.](http://iai.asm.org/)
Macrophage-like cell line MH-S (Fig. 3B) showed that the Fha44-HtrA-producing strain BPSA85 adhered as poorly as the Fha44-producing strain BPSA87, indicating that the fusion of HtrA to Fha44 does not enhance its adhesive properties for these cells.

Antibody response upon infection with recombinant *B. pertussis*. To evaluate the immune responses induced by the recombinant strains, we infected mice i.n. twice at a 2-month interval with approximately $5 \times 10^6$ CFU of the recombinant strain BPSA85 or BPSA199 or the corresponding parental strain BPSA87 or BPRA. Two weeks after the last administration, sera were collected and analyzed for the presence of anti-HtrA and anti-FHA antibodies. A significant serum antibody response was raised against HtrA upon infection with either recombinant strain (Fig. 4A). However, the anti-HtrA IgG titers obtained from mice infected with the FHA-HtrA-producing strain BPSA199 were significantly higher than those obtained from mice infected with the Fha44-HtrA-producing strain.

Significant serum anti-FHA antibody responses were obtained from mice infected with the Fha44-HtrA-producing strain BPSA85 (Fig. 4B). In contrast, and as previously observed (2, 37), no anti-FHA IgG was detected in the sera of mice infected with the corresponding parental strain, BPSA87, indicating that the addition of HtrA to Fha44 enhances the immunogenicity of Fha44. This observation is in contrast to what was observed when HtrA was fused to full-length FHA. The anti-FHA antibody response obtained from mice infected with the FHA-HtrA-producing strain BPSA199 was significantly lower than that obtained from mice infected with the parental counterpart, BPRA (Fig. 4C).

Role of the RGD sequence of HtrA in immune responses. HtrA contains an RGD sequence (26) whose role has not yet been investigated, although the RGD sequences of several microbial proteins have been implicated in several important biological functions (38). FHA also contains an RGD motif, which may be involved in epithelial colonization (15). This motif is not present in Fha44. To investigate whether the RGD motif from HtrA complements the FHA RGD deficiency in Fha44-HtrA, we produced a mutated Fha44-HtrA chimera by introducing a Gly$\rightarrow$Ala change into the HtrA RGD sequence. The resulting chimera was named Fha44-HtrA*, and the recombinant PTX-deficient *B. pertussis* strain producing Fha44-HtrA* was named BPSA167. The lung colonization profile for
this recombinant strain was similar to that obtained with the Fha44-HtrA-producing strain BPSA85 (Fig. 2C), indicating that the HtrA RGD sequence plays no role in the colonization efficacy of the recombinant B. pertussis strains.

However, when the effect of the Gly→Ala change on antibody responses was investigated, the anti-FHA antibody response was greatly enhanced by this substitution. This was already evident 6 weeks after the first administration and was even stronger 2 weeks after the second administration. In contrast, the mutation had no strong effect on the anti-HtrA antibody response (Fig. 5). A statistically significant difference in the anti-HtrA responses of the BPSA85- and BPSA167-infected groups was found for only one time point. These results indicate that the RGD sequence present in the HtrA portion of the Fha44-HtrA chimera is able to modulate the anti-FHA antibody response.

DISCUSSION

Attenuated recombinant B. pertussis has been used successfully to induce local and systemic antibody responses to a variety of foreign antigens after a single i.n. administration (7, 31, 36). For the induction of these immune responses, the heterologous antigens were genetically fused to either full-length or truncated FHA. Although FHA is among the largest bacterial proteins known, it is nevertheless one of the most efficiently secreted proteins from gram-negative bacteria (25). This property, together with its ability to induce strong local and systemic immune responses in B. pertussis-infected humans and in mice, has prompted the use of FHA as a carrier of foreign antigens to be presented at the surfaces of the extracellular milieu. This is in contrast to the very poor production and secretion of a FHA-Sm28GST chimera (36). Since HtrA and Sm28GST contain disulfide bonds, indicating that additional properties of the passenger proteins may strongly influence their ability to be produced and secreted via the FHA secretion machinery.

We have previously described that PTX and FHA exert redundant functions during infection of the mouse respiratory tract (1). PTX- and FHA-deficient strains colonize the respiratory tracts of mice nearly as well as the isogenic wild-type strains. However, when both PTX and FHA are lacking, col-

FIG. 5. Anti-HtrA and anti-FHA antibody responses in serum after i.n. administration of recombinant B. pertussis. Groups of eight mice were infected twice at a 2-month interval with 5 x 10^6 CFU of BPSA85 (black bars) or BPSA167 (white bars). Sera were collected 6 and 8 weeks after the first infection and 2 weeks after the booster, as indicated, and anti-FHA (A) and anti-HtrA (B) IgG titers were estimated by ELISAs using individual sera. * P < 0.05.
onization is strongly impaired. The replacement of FHA with Fha44 in a PTX-deficient background did not restore the ability of the strain to colonize the respiratory tract at wild-type levels (2). Surprisingly, the fusion of HtrA to Fha44 in a PTX-deficient background partially restored the ability of the strain to colonize mouse lungs. This observation suggests that HtrA may at least partially replace some of the functional roles of FHA during colonization of the mouse respiratory tract and may thus act as a colonization factor. HtrA is a heat shock protein. Although heat shock proteins are usually intracellular, some may become surface exposed under certain physiological conditions, and these have been shown to serve as adhesion factors in pathogenic bacteria (10, 11, 13). A potential signal peptide has been identified in HtrA from NTHI, suggesting that it is secreted through the inner membrane of the bacterium and, perhaps, at least partially surface exposed in response to stress (26). As such, it has been suggested that it could act as an attachment factor under certain circumstances (26). Alternatively, the fusion of HtrA may have restored functionality to Fha44, possibly by altering its conformation. This hypothesis is supported by the increase in Fha44 immunogenicity after its fusion to HtrA (see Fig. 5). However, the Fha44-HtrA hybrid was not able to functionally replace full-length FHA in cell adherence assays using epithelial and macrophage-like cell lines, the two cell types that are known to play a role in *B. pertussis* adherence in vivo. Fha44-HtrA-producing *B. pertussis* adhered as poorly to A549 and MH-S cells as did Fha44-producing *B. pertussis* or bacteria that totally lack FHA (2). Although these cell lines are often used to study the in vitro attachment mechanisms of *B. pertussis* to host cells (1, 2), it is possible that the potential adherence activities of HtrA in the context of *B. pertussis* infection of mice may actually involve other cell types or host structures that have yet to be identified. Alternatively, it is possible that the colonization-promoting activity of HtrA depends on functions of the protein other than adherence functions. A role of the protease activity can be ruled out in this context, as care was taken to use an HtrA derivative that is devoid of its natural protease activity. The additional function(s) of HtrA that may be involved in colonization therefore remains unknown at present.

An infection by the *B. pertussis* strain producing the FHA-HtrA protein gave rise to a level of anti-HtrA serum antibodies that was significantly higher than that obtained upon infection with the Fha44-HtrA-producing strain, despite the fact that the Fha44-HtrA chimera was produced and secreted in substantially larger amounts than FHA-HtrA. This observation is in agreement with previous studies showing that although the Sm28GST-containing chimera was very poorly produced and secreted by recombinant *B. pertussis* (36), high levels of anti-SmGST serum antibodies were observed (31). These results suggest that the immunogenicity of the chimera depends on domains of FHA that are missing in Fha44 and that may express adjuvant activities. Alternatively, the carrier may have an effect on the conformation of the passenger protein, which may influence its immunogenicity. In addition, since the FHA-HtrA-producing strain colonized mice significantly better than the Fha44-HtrA-producing strain, the difference in immunogenicity may be a reflection of the difference in colonization between the two recombinant strains.

As previously reported (2), infections with *B. pertussis* strains producing and secreting Fha44 instead of FHA did not result in the induction of significant levels of anti-FHA antibodies. However, when the Fha44-HtrA chimera was produced instead of Fha44, anti-FHA antibodies could readily be detected in the sera of infected mice. Similar results were obtained with a Fha44-TbpB chimera (7), indicating that the fusion of a passenger protein at the C terminus of Fha44 enhances the immunogenicity of the FHA moiety of the hybrid protein. Since two very different passenger proteins had similar effects on the immunogenicity of the carrier protein, it is likely that they induced a conformational change of the carrier, leading to increased immunogenicity. However, in addition, HtrA contains an RGD site that may potentially modulate immune responses via interactions with integrins on antigen-presenting cells (38). A *B. pertussis* strain producing a Fha44-HtrA hybrid protein in which the RGD site of HtrA was altered induced a much higher anti-FHA antibody response than *B. pertussis* producing unaltered Fha44-HtrA, whereas the level of anti-HtrA antibodies was not affected in a dramatic way. These observations point to a complex interplay between the interaction of RGD sequences from surface proteins with their receptors and other functional domains of the proteins. The identification of the cellular and molecular elements involved in this interplay will provide exciting new research areas to help us understand host-pathogen relationships and the induction of specific immune responses.

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