ORIGINAL ARTICLE

A recombinant iron transport protein from *Bordetella pertussis* confers protection against *Bordetella parapertussis*

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ABSTRACT

Whooping cough, which is caused by *Bordetella pertussis* and *B. parapertussis,* is a reemerging disease. New protective antigens are needed to improve the efficacy of current vaccines against both species. Using proteomic tools, it was here found that *B. parapertussis* expresses a homolog of AfuA, a previously reported new vaccine candidate against *B. pertussis.* It was found that this homolog, named AfuA$_{Bpp}$, is expressed during *B. parapertussis* infection, exposed on the surface of the bacteria and recognized by specific antibodies induced by the recombinant AfuA cloned from *B. pertussis* (rAfuA). Importantly, the presence of the O-antigen, a molecule that has been found to shield surface antigens on *B. parapertussis,* showed no influence on antibody recognition of AfuA$_{Bpp}$ on the bacterial surface. The present study further showed that antibodies induced by immunization with the recombinant protein were able to opsonize *B. parapertussis* and promote bacterial uptake by neutrophils. Finally, it was shown that this antigen confers protection against *B. parapertussis* infection in a mouse model. Altogether, these results indicate that AfuA is a good vaccine candidate for acellular vaccines protective against both causative agents of whooping cough.

Key words   *Bordetella parapertussis*, new antigens, vaccine.

Whooping cough, a vaccine-preventable disease, is caused by *B. pertussis* and *B. parapertussis*. Introduction of a wP vaccine, composed of heat-killed *B. pertussis*, has significantly decreased its incidence (1). In the 1990s, concerns about the reactogenicity of wP vaccines led to introduction of aP vaccines composed of purified *B. pertussis* antigens. These acellular vaccines are currently used for both primary vaccination of children and booster immunization of adolescents and adults in an attempt to control spread of these bacteria. Despite this high worldwide vaccine coverage, in the last few decades increased whooping cough outbreaks have been reported in many countries (2–6). Replacement of the wP vaccine by the safer but less effective aP vaccines is thought to have contributed to reemergence of whooping cough. aP vaccines are not only less effective against *B. pertussis* (1), but also do not induce protection against *B. parapertussis* (7); thus, since their introduction, the incidence of whooping cough caused by *B. parapertussis* has increased, as shown by epidemiological studies (8, 9).

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List of Abbreviations: 2D, two dimensional; AfuA, AfuA from *B. pertussis*; AfuA$_{Bpp}$, AfuA from *B. parapertussis*; aP, acellular pertussis vaccine; Bpp∆wbm, O-antigen deficient mutant of *B. parapertussis*; Bppwt, *B. parapertusssis* wild type; FA, Freund's adjuvant; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PMF, peptide mass fingerprints; PMN, neutrophils; rAfuA, recombinant AfuA; SS, Stainer–Scholte; SS-Fe, iron depleted Stainer-Scholte; wP, whole cell pertussis vaccine.

This lack of cross protection is attributable both to antigenic variability between these species (10, 11) and to the presence on the surface of *B. parapertussis* of the O-antigen, which interferes with the eventual opsonic activity of aP vaccine-induced antibodies (7).

Antibody-mediated bacterial phagocytosis by PMNs or macrophages has been found to be critical to inducing bactericidal activity against *B. parapertussis* (12, 13) and protection against infection (7). In the absence of opsonic antibodies, bacterial uptake by these immune cells is not only less efficient but, more importantly, does not result in killing of bacteria (12, 13). Thus, new antigens capable of inducing opsonic activity against *B. parapertussis* may significantly improve the efficacy of whooping cough acellular vaccines.

Antigenic proteins that are expressed during infection and exposed on the surface of *B. parapertussis* may constitute good targets for opsonins. Using a proteomic approach, we have previously detected antigenic proteins exposed on the surface of *B. pertussis* under conditions of iron limitation (14), an environmental condition that human pathogens face during host infection (15). Two of these proteins, AfuA (BP1605) and IRP1-3 (BP1152), which are involved in vital iron uptake systems (16, 17) and expressed in the infective phenotype, proved to be good targets for opsonins and highly protective against infection (18, 19). In the present study, a homolog of AfuA was found to be expressed in iron-starved *B. parapertussis* and its potential as a protective antigen against *B. parapertussis* infection was investigated.

MATERIALS AND METHODS

Bacterial strains and growth conditions

B. parapertussis strain CN2591 and its isogenic mutant strain lacking the O-antigen (BppΔwbm) (20, 21) were used in this study. *B. parapertussis* strains were cultured in SS medium under iron-sufficient (SS) and iron-depleted (SS-Fe) conditions, as previously described (14).

As previously described, *Escherichia coli* BL21-CodonPlus (DE3)-RIL transformed with plasmid pET28/His6-AfuA (19) was used in expression experiments. rAfuA was obtained as described in (19) and adsorbed with sepharose–polymyxin B (Sigma, St. Louis, MO, USA) to eliminate LPS contamination.

Proteomic analysis

B. parapertussis was grown in either SS or SS-Fe medium. Triplicate cultures were harvested and cell lysates prepared and resolved using 2D gel electrophoresis, as previously described (14) with minor modifications.

Briefly, aliquots of cell extracts containing 0.2 mg protein were separated in the first dimension by isoelectric focusing in a Multiphor II Electrophoresis System (GE Healthcare, Piscataway, NJ, USA) for a total of 5 kVh at 20°C using 7 cm linear immobilized pH gradients (pH gradient of 3 to 10). The rehydration solution contained 7 M urea, 2 M thiourea, 33.4 mM dithiothreitol, 1% Triton X-100 and 2% Pharmalyte (broad range, pH 3 to 10). Proteins were separated in the second dimension by using 10% SDS-PAGE and a Bio-Rad Mini-Protean Tetra System (Bio-Rad, Hercules, CA, USA). Gels were stained using colloidal Coomassie blue G250. Spot detection, matching, abundance quantification and normalization were performed using the software Image J. The protein pattern of each growth condition was assessed on the basis of gels from three independent cultures. The protein spots of interest were excised, destained using ammonium bicarbonate in acetonitrile, reduced with dithiothreitol for 30 min, and then digested with trypsin in ammonium bicarbonate overnight (37°C). Peptides were extracted at 37°C using 1:2 (v/v) 5% formic acid/acetonitrile, and reconstituted in 0.1% (v/v) trifluoracetic acid. The resulting digested mixture was then analyzed by Autoflex (Bruker Daltonics, Bremen, Germany) MALDI-TOF-MS. The sample: matrix (α-cyano-4-hydroxycinnamic acid) (1:1) were spotted onto a ground-steel sample target (Bruker Daltonics) and the instrument calibrated using Peptide Calibration Standard (Bruker Daltonics). All samples were analyzed in positive reflector ionization mode (Autoflex II; Bruker Daltonics) and the PMF generated. Protein identification was carried out using the Mascot search engine (22) and Biotools software, version 3.1 (Bruker Daltonics). PMF lists were searched against the NCBI database. The restricting taxonomy frame for the search was set to "other proteobacterias" and the Mascot score probability set for $P < 0.05$ and under the following conditions: peptide mass tolerance, ± 10 ppm; variable modification, carbamidomethylation; and variable modification, oxidation.

Cells

Peripheral blood PMN were isolated from human heparinized venous blood using Ficoll-Histopaque (Sigma) gradient centrifugation, as previously described (23). All procedures involving human samples were in performed accordance with the ethical standards of the 1964 Helsinki declaration and its later amendments, and approved by the Institutional Review Board. Peripheral blood was collected from healthy donors. All individuals provided written informed consent for the collection of samples and subsequent analysis.

Animal experiments

Three to six week old female BALB/c mice were used in animal experiments. The mice were obtained from and bred in the specific pathogen-free breeding rooms of the animal facility of the Faculty of Veterinary, University of La Plata.

Mice were immunized as previously described (19). Briefly, groups of seven BALB/c mice were immunized intraperitoneally with 10 µg of rAfuA protein obtained as previously described (19) and emulsified in complete FA. Twenty one days after the first immunization, a booster dose with the same amount of recombinant protein emulsified in incomplete FA was given. As negative control, seven mice were immunized with an equal amount of adjuvant alone. The mice were bled on Days 0, 21 and 36. Serum was separated by centrifugation and stored at $-20°C$ until analysis.

For challenge, fifteen days after the last immunization, groups of seven immunized mice were sedated and challenged by pipetting 50 µL of PBS containing 1.5×10^6 CFU of *B. parapertussis* grown in SS-Fe onto the external nares, as previously described (24). Three days after challenge, the mice were killed and their lungs excised, homogenized in PBS, serial diluted, plated onto BGA plates supplemented with 15% defibrinated sheep blood (Laboratorio Argentino, Caseros, Argentina) and incubated at 37°C, after which CFU were counted.

Sera from convalescent mice were obtained as previously described (25) with minor modifications. Briefly, groups of four mice were sedated and inoculated by pipetting 50 µL of PBS containing 1.5×10^6 CFU of *B. parapertussis* onto the external nares and serum samples collected on Day 28 post inoculation. Sera from mice inoculated with PBS were used as controls.

Animal handling and all experimental procedures were carried out in compliance with ARRIVE guidelines and in accordance with the European Union Directive for Animal Experiments 2010/63/EU. The animal experiments described in this study were reviewed and approved by the Institutional Animal Care and Use Committee.

ELISA

Antibody titers were determined by ELISA, as previously described (19) with minor modifications. Briefly, twofold diluted samples were assayed in 96-well polystyrene microtiter plates that were coated with 10 µg/mL of recombinant protein in coating buffer (0.5 M carbonate buffer, pH 9.5), wild type *B. parapertussis* grown either in SS or SS-Fe (OD:1) in PBS or Bpp∆wbm grown in SS-Fe (OD:1) in PBS. A HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) was used to detect bound IgG. For measuring IgG isotypes, amounts of bound antibody were determined using HRP-labeled subclass-specific anti-mouse IgG1 or IgG2a (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Titer, calculated by an endpoint method (26), was defined as the reciprocal of the highest serum dilution that gave a reading above the cutoff.

Phagocytosis assay

Phagocytosis of *B. parapertussis* was evaluated as previously described (12) with minor modifications. Briefly, wild type *B. parapertussis* or Bpp∆wbm grown in SS-Fe were opsonized by 30 min incubation at 37°C with either rAfuA-induced or naïve mouse serum. Opsonized bacteria were washed and further incubated with PMN for 10 min at 37°C to allow interaction (MOI 50), extensively washed at 4°C to remove non-attached bacteria and further incubated for 40 min at 37°C. Phagocytosis was stopped by placing PMN on ice. Cells were fixed using 4% paraformaldehyde, washed once with PBS and incubated for 10 min at room temperature with PBS containing 50 mM NH$_4$Cl. PMN surface-bound bacteria were detected by a two-step antibody dependent labeling procedure as follows. PMN were incubated with polyclonal rabbit anti-*B. parapertussis* antiserum (30 min at 4°C), followed by incubation with cyanine 3-conjugated goat F(ab')$_2$ fragments of anti-rabbit immunoglobulin (Jackson ImmunoResearch) for another 30 min at 4°C. To determine the number of intracellular bacteria, cells were washed and permeabilized by incubation with PBS containing 0.1% saponin (Sigma) and 0.2% BSA for 30 min, and further incubated for other 30 min with rabbit anti-*B. parapertussis* antiserum in the presence of 0.1% saponin and 0.2% BSA. After washing three times, PMN were incubated for 30 min with FITC-conjugated F(ab')$_2$ fragments of anti-rabbit immunoglobulin (Jackson ImmunoResearch). Bacterial phagocytosis by PMN was evaluated by fluorescence microscopy using a confocal laser scanning microscope (Leica TCS SP5; Leica, Heidelberg, Germany). The number of extracellular (red and green fluorescent) and intracellular bacteria (green fluorescent) per cell was determined by microscopic examination of at least 10 randomly selected fields with a minimum of five cells per field. To avoid eventual cytophilic binding of antibodies to FcγR, all incubations were done in the presence of 25% heat-inactivated human serum. All experiments were carried out at least twice in triplicate.

Immunoblot analysis

Purified rAfuA, whole cell lysates or outer membrane fractions from *B. parapertussis* obtained as previously

described (14), were prepared with Laemmli sample buffer and run on 10% SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (Immobilon PVDF Millipore) sheets and incubated with sera from mice immunized with rAfuA. Immunochemical detection was performed using alkaline phosphatase-conjugated goat antimouse IgG (Jackson Immuno Research).

Statistical analysis

Differences between the results of the experiments were evaluated by mean of ANOVA or Student's *t*-test. Significance was accepted at $P < 0.05$. Results are shown as means and SD.

RESULTS

B. parapertussis expresses AfuA

Two-dimensional gel electrophoresis was used to investigate expression of AfuA in *B. parapertussis* by analyzing whole cell lysates of the bacteria cultivated under SS-Fe or SS conditions. Considering the isoelectric points and molecular weights of the predicted AfuA (8.6 and 37.46 kDa, respectively), a number of proteins were targeted for identification in each gel. The spots were submitted to tryptic digestion and MALDI-TOF-MS. Two of them (shown in Fig. 1) were identified as AfuA (BPP2980), hereafter called $AfuA_{Bpp}$. As observed for AfuA of *B. pertussis*, $AfuA_{Bpp}$ was more abundant in iron-starved bacteria (spot value SS/SS-Fe: 0.310/3.118).

Antibodies induced by rAfuA recognize $AfuA_{Bpp}$

Western blot analysis of whole cell lysates and outer membrane fractions of *B. parapertussis* cultivated under SS or SS-Fe growth conditions showed that antibodies raised against rAfuA recognized only one protein of the molecular weight of AfuA in each sample (Fig. 2a), indicating that antibodies raised against *B. pertussis*

recombinant AfuA recognize the homolog in *B. parapertussis*. As expected, they further show that $AfuA_{Bpp}$ is present in the outer membrane fractions of *B. parapertussis*.

Whole cell ELISA using live iron-starved or iron-replete *B. parapertussis* as antigen was used to determine AfuA antibody titers to evaluate whether $AfuA_{Bpp}$ is exposed on the bacterial surface. Sera from mice immunized with recombinant AfuA showed bacterial recognition in both assays; however, titers were significantly higher in the ELISA in which iron-starved bacteria were used as coating antigen (Fig. 2b). These results not only confirm that $AfuA_{Bpp}$ is iron-regulated, but also indicate that $AfuA_{Bpp}$ is exposed on the surface of the bacteria. Next, whether the O-antigen interferes with antibody recognition of $AfuA_{Bpp}$ was evaluated. To this end, whole-cell ELISA using an O-antigen-deficient mutant strain of *B. parapertussis* cultivated under iron starvation as coating antigen was performed in parallel. No significant differences were observed between $AfuA_{Bpp}$ recognition of the wild type and O-antigen mutant strain of *B. parapertussis* (Fig. 2b), indicating that the O-antigen has little or no effect on antibody recognition of $AfuA_{Bpp}$ on the bacterial surface.

Anti-rAfuA antibodies promote PMN phagocytosis of *B. parapertussis*

Antibody recognition as determined by ELISA does not necessarily indicate biological activity. Therefore, whether the antibodies induced by the recombinant protein were able to induce opsonophagocytosis of *B. parapertussis* by human PMN was then examined. Figure 3 shows that anti-rAfuA antibodies significantly increased PMN phagocytosis of iron-starved *B. parapertussis* as compared with naïve serum. Again, the presence of the O-antigen did not interfere with the biological activity of rAfuA-induced antibodies, as indicated by the lack of significant difference between opsonophagocytosis of the O-antigen deficient mutant and the wild type strain of *B. parapertussis* (Fig. 3).
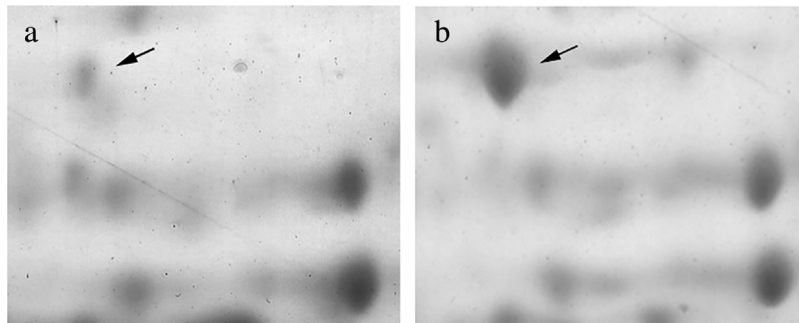

Fig. 1. 2D gel image showing $AfuA_{Bpp}$ protein spots. Image J software was used for protein spot detection, matching and quantification in 2D gels of *B. parapertussis* growth in (a) SS and (b) SS-Fe. The protein spots indicated with arrows were in-gel digested and analyzed by MALDI-TOF-MS to create PMFs and the PMFs identified by using MASCOT and Biotools software (MS/MASCOT score, 212; coverage, 26%).
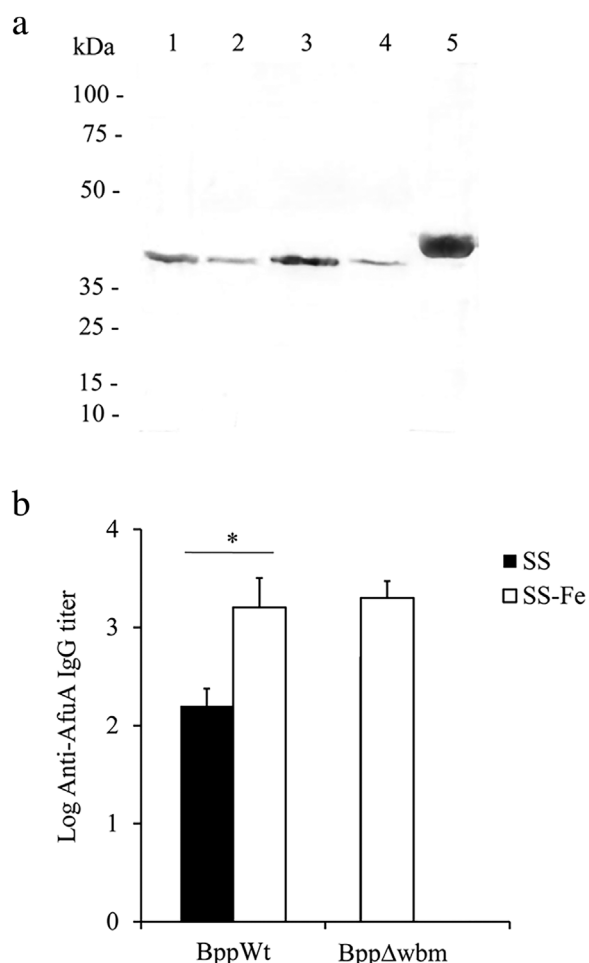
a

b

Fig. 2. Antibodies induced by rAfuA recognize AfuA$_{Bpp}$. (a) Whole cell lysates (Lanes 1 and 2) and outer membrane proteins (Lanes 3 and 4) from *B. parapertussis* grown either under iron starvation (Lanes 1 and 3) or iron excess (Lanes 2 and 4) cultural conditions were subjected to SDS-PAGE (10%) and immunoblot analysis using anti-rAfuA antibodies. rAfuA was run as positive control (Lane 5). The gel was loaded with equivalent ODs in Lanes 1 and 2, and the same amount of protein in Lanes 3 and 4. (b) Antibody titers induced in mice by immunization with rAfuA were determined by whole cell ELISA. Plates were coated with either iron-starved BppΔwbm or Bppwt grown under iron-replete or iron-depleted conditions. Sera from mice immunized with rAfuA showed significantly stronger reactions with iron-starved Bppwt than with Bppwt grown under iron-replete conditions (*$P < 0.05$). Antibody titers are expressed as the mean of the log of the end point titer of each serum ± SD.

AfuA$_{Bpp}$ is expressed during infection

To investigate whether AfuA$_{Bpp}$ is expressed during infection, the presence of antibodies against AfuA was evaluated in sera from mice convalescing from *B. parapertussis* infection. To that end, sera from naïve and convalescent mice were analyzed by ELISA using purified rAfuA as coating antigen. Only sera from infected animals showed significant amounts of anti-AfuA antibodies (Fig. 4) suggesting that AfuA$_{Bpp}$ is expressed during infection.

rAfuA protects mice against *B. parapertussis* infection

Antibody responses induced in mice immunized twice with rAfuA were investigated. Mice injected with the adjuvant alone served as control. Immunization with rAfuA induced an IgG specific response that was already detectable 21 days after the first immunization. As previously found (19), repeated immunization led to a booster response of specific IgG anti-rAfuA (log titer: 6.7 ± 0.25) with a balanced IgG1/IgG2a response (Fig. 5).

To investigate whether rAfuA can protect against *B. parapertussis* infection, mice immunized with rAfuA were challenged intranasally with iron-starved *B. parapertussis*. Mice immunized with FA alone were used as negative controls. Protective activity was evaluated by determining bacterial clearance in the lungs. Control mice vaccinated with adjuvant alone had mean loads around 10^7 CFU in the lungs 3 days post challenge. Mice given rAfuA plus adjuvant exhibited significant protection ($P < 0.05$) against *B. parapertussis* as compared with mice immunized with adjuvant alone (Table 1).

DISCUSSION

Despite widespread vaccination programs focused on whooping cough control, a resurgence of the disease has been reported in the last few decades (2–6). Although both *B. pertussis* and *B. parapertussis* can cause this disease, efforts to improve preventive strategies have been mainly focused on controlling circulation of *B. pertussis*. However, several studies have suggested an increasing incidence of *B. parapertussis* worldwide (8, 9, 27). This increase has been mainly attributed to the introduction of aP vaccines that confers no protection against this species (4, 7, 8). It has been shown by individual evaluation in a mouse model that, except for fimbriae (28), none of the aP vaccine components confer protection against *B. parapertussis* (10). Even in the case of fimbriae, the level of protection against *B. parapertussis* infection is reportedly lower than against *B. pertussis* infection (28). Two possible causes for this lack of cross protection have been identified. One of them is the immunogenic variability of aP components between the *Bordetella* species (10, 11, 28). The other is the presence of the O-antigen on *B. parapertussis* surface.
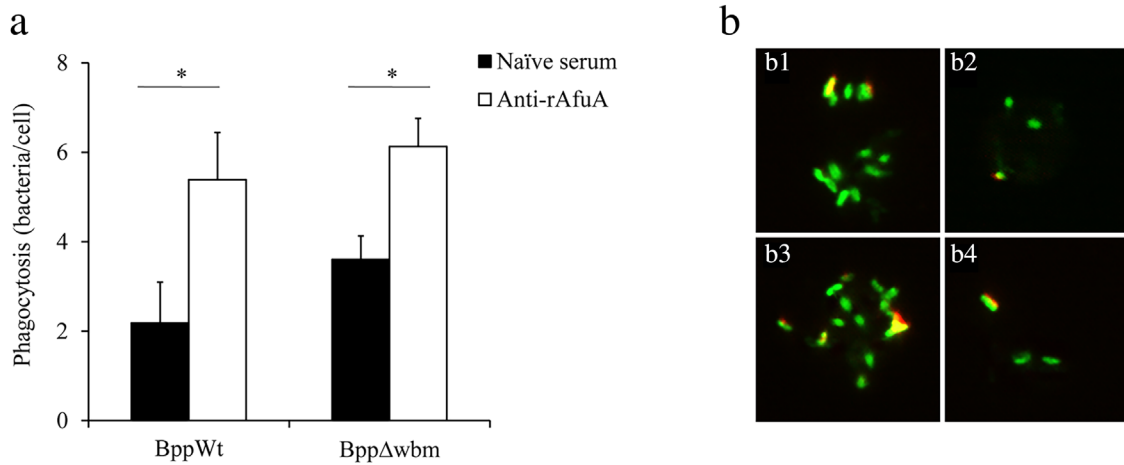
a

b

Fig. 3. *B. parapertussis* opsonophagocytosis induced by anti-rAfuA antibodies. (a) Bppwt or BppΔwbm grown under iron-depleted conditions were incubated with anti-rAfuA or naïve serum for 30 min at 37°C, washed, and further incubated with PMN (MOI 50) for 10 min at 37°C. After attachment, PMN were washed and further incubated for 40 min at 37°C to allow internalization. Cells were fixed and intracellular bacteria labelled with green and extracellular bacteria with both green and red fluorescent dyes. Bacterial phagocytosis was assessed by confocal laser scan fluorescence microscopy. At least 50 cells per slide were counted. PMN phagocytosis of anti-rAfuA opsonized Bppwt and anti-rAfuA opsonized BppΔwbm differed significantly from PMN phagocytosis of naïve serum opsonized Bppwt and BppΔwbm, respectively (*$P < 0.05$). Data represent the mean ± SD of two independent experiments with PMN from different donors. (b) Confocal fluorescence microscopy of PMN incubated 40 min at 37°C with (b1, b2) Bppwt or (b3, b4) BppΔwbm opsonized with (b1, b3) anti-rAfuA or (b2, b4) naïve serum. Representative panels of one of two independent experiments are shown.

Zhang *et al.* have shown that the O-antigen interferes with bacterial recognition by aP induced antibodies (7). However, immunization of mice with aP vaccine does not protect them against infection with an O-antigen deficient *B. parapertussis* mutant strain (7), indicating
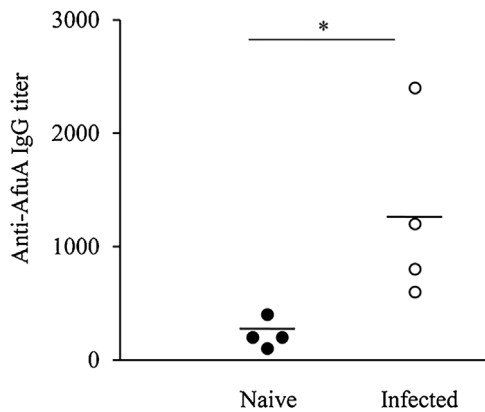

Fig. 4. AfuA is expressed during *B. parapertussis* infection. Anti-AfuA antibody titers induced in mice infected with *B. parapertussis* were determined by ELISA. Plates were coated with rAfuA as antigen. Sera of infected mice had significantly higher anti-AfuA antibody titers than sera of naïve mice (*$P < 0.05$). Antibody titers are expressed as the end point titer of each serum. The respective mean titer of each group is shown.

that other factors, such as antigenic variability, are involved in failure of aP vaccines against *B. parapertussis*.

Clinical and epidemiological data show the need for better preventive strategies against whooping cough, including vaccines that perform better against both *B. pertussis* and *B. parapertussis*. To our knowledge, only a few antigens have been proposed as potential acellular vaccine candidates against *B. parapertussis*. Komatsu *et al.* showed that immunization with *B. parapertussis* filamentous hemagglutinin protects against infection and proposed development of acellular vaccines against *B. parapertussis* composed of filamentous hemagglutinin and other antigens purified from this pathogen (29). Other studies have suggested O-antigen as a candidate to be included in current pertussis vaccines (25). However, to our knowledge, this antigen has not yet been evaluated.

We previously used a comparative proteomic analysis to search for new vaccine components able to improve immunity against *B. pertussis* (14) and identified an iron-repressed protein, named AfuA (BP1605), expressed during infection that proved to be protective against this pathogen (19). In the present study, we analyzed *B. parapertussis* response to iron starvation and observed that expression of BPP2980, the homolog of *B. pertussis* AfuA, is also induced under this environmental condition. This protein, named AfuA$_{Bpp}$, has considerable sequence similarity (99.4% of identity) with AfuA of
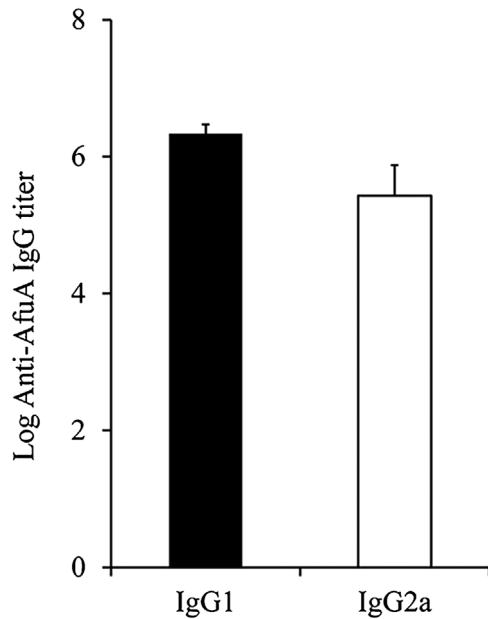
Fig. 5. Anti-rAfuA antibody isotype profile. Anti-rAfuA IgG1 and IgG2a antibody titers were determined by ELISA 15 days after booster immunization with rAfuA (Day 36). Antibody titers are expressed as the mean of the log of the end point titer of each serum ± SD.

B. pertussis. The differences between the species are in residues 31 and 149. According to bioinformatics analysis, only residue 31 may be part of an epitope. In particular, it may be part of one of the 18 linear B-cell predicted epitopes (30). The reported crystal structure of BP1605 (Protein Data Bank code 1Y9U) includes only the residue 149 and shows that this residue is neither surface-exposed nor part of any structure-based epitope, as predicted with discotope (31). Altogether, these results suggest that there is low antigenic variability between *B. pertussis* and *B. parapertussis* AfuA. In agreement with these findings, we observed that antibodies induced by recombinant AfuA cloned from *B. pertussis* not only recognized AfuA$_{Bpp}$ in western blot analysis of both whole cell lysates and outer membrane

fractions of *B. parapertussis*, but also recognized the protein on bacterial surfaces, as determined by whole cell ELISA. We confirmed the biological activity of rAfuA-induced antibodies in opsonophagocytosis studies using freshly isolated human neutrophils. Our results indicate that antibodies raised against rAfuA are able to opsonize *B. parapertussis* and promote bacterial phagocytosis by neutrophils, confirming not only surface exposure of the AfuA$_{Bpp}$, but also recognition of the native protein by rAfuA-induced antibodies. Importantly, we observed that the O-antigen does not hamper antibody recognition of AfuA$_{Bpp}$. The presence of the O-antigen did not interfere with antibody detection of AfuA$_{Bpp}$ in either ELISA or in functional studies.

Both neutrophils and opsonic antibodies are reportedly crucial to controlling *B. parapertussis* infection (32). According to previous studies, this pathogen is able to survive innate interactions with neutrophils (12, 33) and macrophages (13). In the absence of opsonins, phagocytosed *B. parapertussis* remains viable inside both types of immune cells and eventually replicates inside macrophages, which may constitute an intracellular niche of persistence (12, 13). Currently available acellular vaccines do not induce opsonic antibodies against *B. parapertussis* (7). Of the antigens present in aP vaccines, pertactin is the only one able to induce opsonins (34). In addition to the O-antigen effect on antibody recognition (7) and the reported antigenic variability between *B. pertussis* and *B. parapertussis* pertactin (10), in the last few years *B. parapertussis* circulating strains that do not express this antigen have emerged (35, 36). We have previously found that antibodies raised against rAfuA are able to opsonize *B. pertussis* and promote efficient bacterial phagocytosis by human neutrophils (19). In the present study, we showed that anti-rAfuA antibodies induced by recombinant AfuA cloned from *B. pertussis* are also able to opsonize *B. parapertussis* and promote bacterial uptake by neutrophils, which adds value to AfuA as a potential candidate for whooping cough control. Confirming the relevance of AfuA as a promising new protective immunogen, immunization of mice with rAfuA induced significant protection against *B. parapertussis* proliferation in the lungs as compared with control mice.

In summary, better preventive approaches against whooping cough should include new vaccines protective against *B. parapertussis*. Previous studies have pointed out AfuA as a potential candidate for improving vaccines against *B. pertussis*. The data presented in this study indicate that this protein also confers protection against *B. parapertussis*, shedding new light on the potential of this antigen as a promising candidate for improving vaccines against whooping cough.

Table 1. Protection against *B. parapertussis* infection induced by rAfuA immunization

Vaccine	Log CFU in lungs†	Units of protection‡
rAfuA/FA	5.96 ± 0.24§	0.97
PBS/FA	6.93 ± 0.06	—

†Content of bacteria in lungs is presented as the mean log CFU ± SD per group; ‡units of protection were obtained by subtracting the mean log CFU of the vaccinated group from the mean log CFU of the control group; §significantly different from FA-immunized mice ($P < 0.05$).

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DISCLOSURE

The authors of this manuscript have no financial conflicts of interest related to the studies described herein.

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