Vaccine 31 (2013) 3543–3548

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Bordetella pertussis iron regulated proteins as potential vaccine components

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ARTICLE INFO

Article history:
Received 9 November 2012
Received in revised form 27 March 2013
Accepted 17 May 2013
Available online 31 May 2013

Keywords:
Bordetella pertussis
Vaccine
New antigens

ABSTRACT

Bordetella pertussis is the etiologic agent of whooping cough, an illness whose incidence has been increasing over the last decades. Pertussis reemergence despite high vaccination coverage, together with the recent isolation of circulating strains deficient in some of the vaccine antigens, highlight the need for new vaccines. Proteins induced under physiological conditions, such as those required for nutrient acquisition during infection, might represent good targets for better preventive strategies. By mean of serological proteome analysis we identified two novel antigens of *B. pertussis* potentially involved in iron acquisition during host colonization. We had previously demonstrated that one of them, designated IRP1-3, is protective against pertussis infection in mice. In the present study, we show that the other antigen, named AfuA (BP1605), is a highly antigenic protein, exposed on the bacterial surface, conserved among clinical isolates and expressed during infection. Immunization of mice with the recombinant AfuA induced opsonophagocytic antibodies which could explain the protection against *B. pertussis* infection conferred by mice immunization with rAfuA. Importantly, we found that the addition of rAfuA and rIRP1-3 proteins to the commercial three pertussis components acellular vaccine significantly increased its protective activity. Taken together, our results point at these two antigens as potential components of a new generation of acellular vaccines

1. Introduction

Whooping cough or pertussis is a highly contagious disease of humans caused mainly by *Bordetella pertussis* [1]. Upon the introduction of the whole cell pertussis vaccine there was a significant decrease in the number of pertussis cases. The side effects of these kind of vaccines led to the development of a new generation of less reactogenic acellular pertussis (Pa) vaccines consisting of different combinations of *B. pertussis* virulence factors. All of them expressed in the virulent phase of the bacteria positively regulated by the BvgAS two-component phosphorelay system [2,3]. The BvgAS system mediates the transition between virulent (Bvg+), and avirulent (Bvg−) phases of *B. pertussis* in response to environmental signals, such as temperature among others [4,5]. Despite vaccination, many industrialized countries have had an increase of pertussis reported cases in the past decades, mostly among adolescents and adults [6–8]. The reemergence of pertussis has been attributed to various factors, including waning vaccine-induced immunity, suboptimal vaccine formulations and changes in circulating strains suggesting vaccine-induced adaptation. Remarkably, recent studies reported circulating strains of *B. pertussis* lacking the expression of two of the main protective vaccine antigens, pertactin and pertussis toxin, due to genotypic changes [9–11]. These findings, together with epidemiological evidence suggest that currently available acellular vaccines may not provide efficient protection against circulating strains.

Iron is a crucial factor for bacterial colonization [12]. Successful microbial pathogens have developed mechanisms to overcome host iron restriction [13,14]. Several observations suggest that *B. pertussis* is iron-starved inside its human host and responds by expressing a number of vital iron uptake systems [15–18]. With the aim of identifying protective antigens, we have focused on proteins that might be necessary for this nutrient acquisition during colonization. Using comparative proteomics we identified several *B. pertussis* proteins differentially induced by iron starvation [19]. Among them, two proteins acknowledged as putative iron binding proteins were recognized by human antibodies induced by *B. pertussis* infection [19]. We previously showed that one of these, named IRP1-3, is highly immunogenic and protective against *B. pertussis* infection in monovalent formulation [20]. The other protein, BP1605 (named as AfuA) has significant homology with iron transport proteins of other bacteria, such as FbpA from *Neisseria*

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http://dx.doi.org/10.1016/j.vaccine.2013.05.072

meningitidis [21], HitA from *Haemophilus influenza* [22], or FbpA from *Campylobacter jejuniv* [23]. Interestingly, FbpA was found able to elicit bactericidal antibodies against *N. meningitidis* [24]. BP1605 has been recently proposed as an iron binding protein required for the utilization of transferrin and lactoferrin, the two main iron sources *in vivo* [25], suggesting a key role during infection. In this study we investigated the protective capacity of AfuA alone, and the effect of the addition of AfuA and IRP1-3 on the protective activity of a three-pertussis-components DTPa vaccine in use.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. pertussis strains BP536, a streptomycin-resistant derivative of Tohama I, and BP537, a Bvg-phase locked derivative of Tohama I [26] were used in this study. Clinical strains isolated from Argentinean patients in the period 2002–2007 were used for PCR and western blot analysis. *B. pertussis* strains were cultured under iron starvation and iron sufficient conditions as previously described [19].

2.2. Cells

Human peripheral blood neutrophils (PMN) were isolated from heparinized venous blood using Ficoll-Histopaque (Sigma, St. Louis, MO) gradient centrifugation as previously describe [27].

2.3. Antigens production

The open reading frame of AfuA was cloned in the pET28a vector (Novagen, Madison, WI). Briefly, specific primers for *afuA* gene sequence were designed with *BamHI* and *HindIII* restriction sites (primers: AAGGATCCCCGTCGACCAAGCGTTC and ACCTAAGCTTTCAGTTGTCGAAGCCCACC). *B. pertussis* genomic DNA was used as template for PCR with Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA). The PCR product corresponding to the full-length gene sequence of *afuA* was cloned into the *BamHI* and *HindIII* restriction sites of the pET28a (Novagen, Madison, WI) expression vector to generate the recombinant protein with an N-terminal histidine tag. The recombinant AfuA was cloned, produced and purified in *Escherichia coli* strain BL21-CodonPlus (DE3)-RIL as described [20]. The recombinant IRP1-3 was produced as in [20]. rAfuA and rIRP1-3 were adsorbed with Sepharose-polymyxin B (Sigma, St. Louis, MO) to eliminate lipopolysaccharide contamination.

2.4. Mice immunization and challenge

Three to 6 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 School of Veterinary, University of La Plata. Groups of 10 mice were immunized intraperitoneally (i.p.) with 2 μg of rAfuA emulsified in complete Freund's adjuvant, 1/5 of the human dose of commercial DTPa vaccine *Infanrix*™ (GSK, Rixensart, Belgium), or 1/5 of DTPa vaccine plus 2 μg of rAfuA, 2 μg of rIRP1-3, or 2 μg of both rAfuA and rIRP1-3. A booster dose was given on day 21 with the respective vaccine. In the case of the vaccine containing 2 μg of rAfuA alone, the protein was emulsified in incomplete Freund's adjuvant for booster vaccination. As negative controls groups of 10 mice were immunized with either Freund's adjuvant or Alum. Mice immunized with Alum were included as control of DTPa vaccine *Infanrix*™ either alone in combination with rIR1-3 and/or rAfuA. The mice were bled at days 0, 21 and 36 and the serum was separated and stored at −20 °C. Animal handling and all procedures were carried out in compliance with the recommendations of the "Guide for the Care and Use of Laboratory Animals" of the National Research Council (Academy Press, 1996, Washington).

Fifteen days after the last immunization mice were challenged by pipetting 50 μl of PBS containing 1.5×10^6 CFU of *B. pertussis* grown in SS-Fe onto the external nares as described [28]. Three and eight days after challenge, mice were sacrificed and the lungs were excised. The lungs were homogenized in PBS, serial diluted, plated onto BGA plus 15% sheep blood, and CFU were counted after incubation at 37 °C.

2.5. ELISAs

The antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) as previously described [20]. 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), or *B. pertussis* grown either in SS (Bp + Fe) or SS-Fe (Bp-Fe) (OD:1 in PBS). Bound IgG was detected with horseradish peroxidase (HRP)-conjugated goat anti-human IgG, or HRP-conjugated goat anti-mouse IgG (both from Jackson ImmunoResearch, Baltimore Pike). For measuring IgG isotypes, detection of bound antibody was determined using HRP-labeled subclass-specific anti-mouse IgG1 or IgG2a (Santa Cruz Biotechnology, Santa Cruz, CA).

2.6. Phagocytosis

Opsonization was evaluated as described before [27]. Briefly, Bp-Fe were opsonized by 30 min incubation at 37 °C with either rAfuA-induced or naïve serum in a final volume of 90 μl. Opsonization was evaluated by incubation of serum opsonized bacteria with phycoerythrin (PE)-labeled goat $F(ab')_2$ fragments of anti-mouse IgG (Southern Biotechnology, Birmingham, AL) for 30 min at 4 °C. FACS analysis was used to evaluate opsonization as described [27]. Bacterial phagocytosis was evaluated by confocal microscopy as in [29]. Opsonized bacteria were incubated with PMN at a multiplicity of infection (MOI) of 30 bacteria per cell for 15 min at 37 °C to allow bacterial interaction with PMN, washed to remove non-attached bacteria, and further incubated for 1 h at 37 °C. Phagocytosis was evaluated by two color microscopic analyses using a confocal laser scanning microscope (Leica TCS SP5, Germany) as previously described [29]. The number of extracellular (red and green fluorescent) and intracellular bacteria (green fluorescent) per cell was determined by microscopic examination of 20 randomly selected fields showing a minimum of 5 cells per field.

2.7. Immunoblot analysis

Purified rAfuA, bacterial lysates, and filtered supernatants of Bp-Fe cells incubated for 30 min at either 56 °C or room temperature were prepared with Laemmli buffer, and ran on 10% SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (Immobilon PVDF Millipore) sheets and incubated with mouse anti-rAfuA. The immunochemical detection was performed using alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immuno Research, Baltimore Pike).

2.8. Statistical analysis

Differences between the results of the experiments were evaluated by mean of ANOVA or *t*-test. Significance was accepted at $P < 0.05$. Results are shown as means and SD.

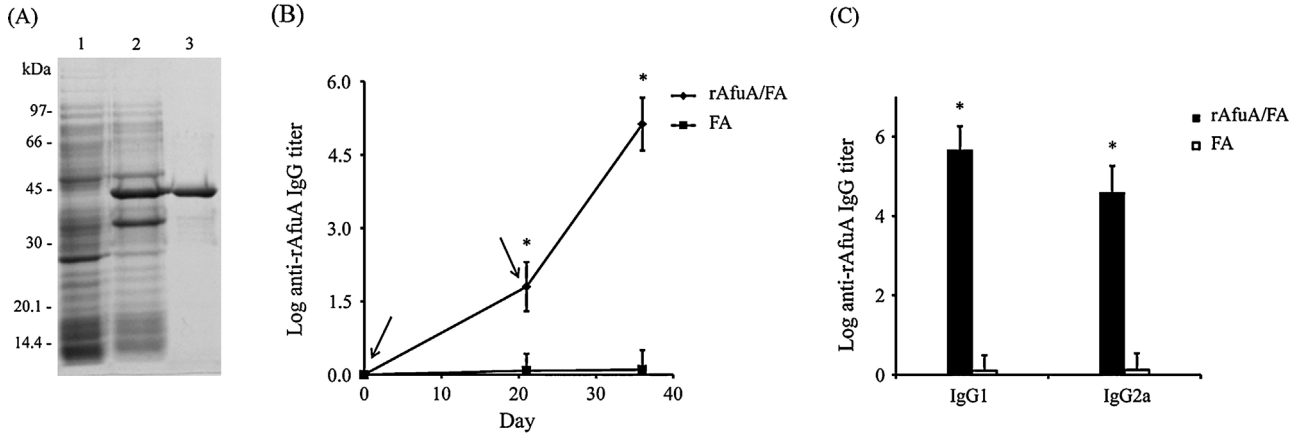

Fig. 1. AfuA production and antibody response. (A) Whole cell proteins of uninduced *E. coli* (lane 1), induced *E. coli* (lane 2), or purified rAfuA (lane 3) were separated by SDS-PAGE (10%) and stained with Coomassie blue. (B) Time course of type-specific anti-rAfuA IgG antibody response after priming and booster immunization with rAfuA. Groups of ten mice were immunized on days 0 and 21 (indicated by arrows). Serum samples were taken on days 0, 21, 36, and antibody response was determined by ELISA. Serum of mice immunized with rAfuA had significantly higher anti-rAfuA antibody titers than the respective controls (*$P < 0.05$). Serum sample of day 36 had significantly higher anti-rAfuA antibody titers than serum samples of day 21 (**$P < 0.05$). Data represent mean antibody titers ± SD. (C) Anti-rAfuA IgG1 and anti-rAfuA IgG2a antibodies were determined by ELISA. Sera of mice immunized with rAfuA had significantly higher anti-rAfuA IgG1 and anti-rAfuA IgG2a antibody titers than the respective controls (*$P < 0.05$). The data show mean antibody titers ± SD at day 36.

3. Results

3.1. AfuA is immunogenic in mice

In order to examine the immunogenicity of AfuA we cloned, expressed, and purified recombinant AfuA (rAfuA) from *E. coli* (Fig. 1A). Immunization with rAfuA elicited high levels of anti-rAfuA IgG after the first immunization. Repeated immunization led to a booster response (Fig. 1B). To investigate the type of immune response induced by rAfuA we further determined the profile of the evoked humoral responses as a marker of the elicited cellular immune responses (IgG2a, T helper 1 [Th1]; IgG1, T helper 2 [Th2]) [30]. Fig. 1C shows that animals immunized with rAfuA/FA elicited both IgG1 and IgG2a response against rAfuA.

3.2. AfuA is exposed on the bacterial surface

We have previously found AfuA in the outer membrane subproteome [19]. To get information about the surface exposure of AfuA we performed an ELISA using heat inactivated iron-starved whole cell bacteria (HBp-Fe) as antigen. Sera from mice immunized with the recombinant protein showed bacterial recognition suggesting that AfuA is exposed on the surface of the bacteria (Fig. 2A). Cross-reactivity with other bacterial protein was ruled out by western blot analysis. Fig. 2B shows that anti-rAfuA sera recognized only one band of the molecular weight of AfuA. Since many surface proteins are labile and detached from the bacteria by heat treatment, we next evaluated the same sera using live bacteria as antigen. Fig. 2A shows a significant increase ($P < 0.05$) of antibody recognition when live iron-starved *B. pertussis* was used as coating agent. Western blot analysis of the extracellular medium of Bp-Fe incubated for 30 min at 56 °C, confirmed the release of AfuA by heat treatment (Fig. 2C).

3.3. Anti-rAfuA antibodies promote PMN phagocytosis of B. pertussis

We next examined whether the antibodies induced by the recombinant protein were able to induce bacterial phagocytosis. Fig. 3A shows that iron starved *B. pertussis* was efficiently opsonized

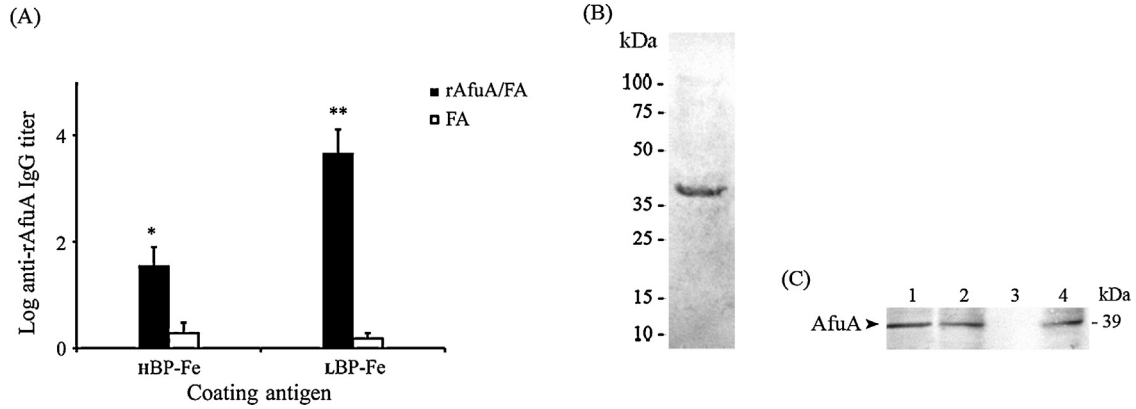

Fig. 2. Bacterial surface exposure of AfuA. (A) Anti-rAfuA antibodies titers induced in mice immunized with rAfuA/FA or FA alone were determined by whole cell ELISA. Plates were coated with either heat inactivated Bp-Fe (HBp-Fe) or live Bp-Fe (LBp-Fe) as antigens. Sera of mice immunized with rAfuA/FA had significantly higher anti-rAfuA antibody titers than sera of mice immunized with FA (*$P < 0.05$). Sera of mice immunized with rAfuA showed a significant higher reaction with LBp-Fe than with HBp-Fe (**$P < 0.05$). The figure shows mean antibody titers ± SD. (B) Whole cell lysate from Bp-Fe was ran in SDS-PAGE (10%), and transferred to PVDF membrane. Immunoblot analysis was performed with polyclonal mouse antiserum raised against rAfuA. (C) Cell lysates of Bp-Fe incubated 30 min at room temperature (lane 1), Bp-Fe incubated 30 min at 56 °C (lane 2), supernatant of Bp-Fe incubated 30 min at room temperature (lane 3), and supernatant of Bp-Fe incubated 30 min at 56 °C (lane 4) were subjected to SDS-PAGE (10%) and immunoblot analysis using anti-rAfuA antibodies. The gel was loaded with the same amount of proteins of each sample.

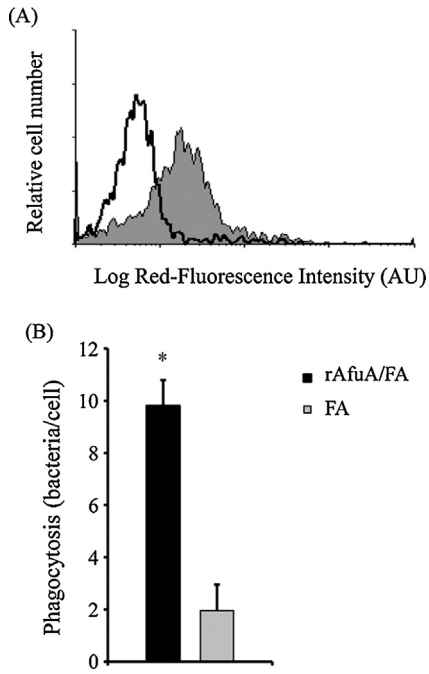

Fig. 3. *B. pertussis* opsonization by anti-rAfuA and bacterial phagocytosis by PMN. (A) Opsonization of *B. pertussis* by anti-rAfuA antibodies. Bp-Fe were incubated with anti-rAfuA serum (gray filled) or naïve serum (black line), and further stained with PE-labeled goat $F(ab')_2$ fragments of anti-mouse IgG. Bacterial red fluorescence association was determined by flow cytometry. The figure shows representative histograms of three independent studies. (B) PMN phagocytosis of *B. pertussis* opsonized with anti-rAfuA. Bp-Fe were incubated with anti-rAfuA or naïve serum, washed, and further incubated with PMN (MOI of 30) for 15 min at 37 °C. After attachment, PMN were washed and further incubated for 1 h at 37 °C to allow internalization. Cells were fixed, and permeabilized prior to labeling intracellular bacteria in green and extracellular bacteria with both green and red fluorescent dyes. Bacterial phagocytosis was assessed by confocal laser scan fluorescence microscopy. To assess the number of phagocytosed bacteria at least 100 cells were counted per slide. The data represent the mean ± SD of four experiments with PMN from different donors. Phagocytosis of anti-rAfuA opsonized Bp-Fe by PMN differed significantly from PMN phagocytosis of Bp-Fe incubated with naïve serum (*$P < 0.05$). Data represent the mean ± SD of 3 independent experiments with PMN of different donors.

by anti-rAfuA antibodies which eventually increased the efficiency of PMN uptake of Bp-Fe by PMN compared to naïve serum (Fig. 3B).

3.4. AfuA is conserved in clinical isolates and expressed during infection

AfuA gene conservation was investigate in 14 randomly chosen clinical isolates that originate from a survey of *B. pertussis* in Argentine, from 2002 to 2007. Primers based on *B. pertussis* Tohama I were designed and used for PCR amplification from each clinical isolate. The gene was found present in all the 14 clinical isolates tested (Fig. 4A), the PCR products were of the expected size (1050 bp), and no sequence variation was detected (100% identity at gene level, data not shown). Immunoblot analysis demonstrated that five isolates randomly chosen expressed AfuA under iron limitation (Fig. 4B). Finally, anti-AfuA antibodies determination by ELISA showed high titers of specific antibodies against AfuA in pooled sera from infected individuals (1500 ± 80) and undetectable levels in sera from non-infected individuals, suggesting that AfuA is expressed during infection.

3.5. AfuA expression is independent of the virulence state of the bacteria

Western blot analysis of *B. pertussis* (BP536) grown under iron starvation with or without the addition of modulating

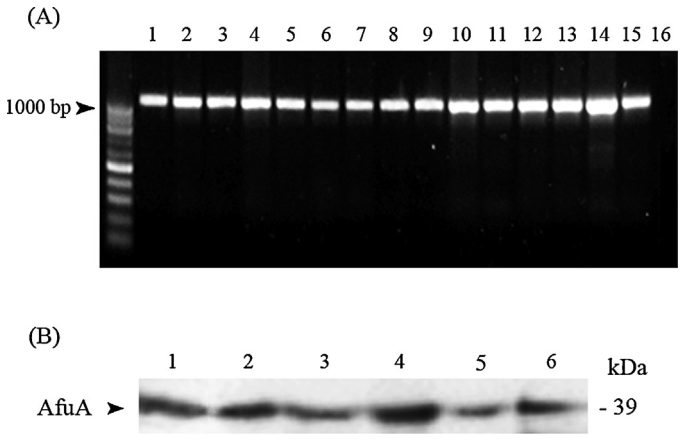

Fig. 4. Clinical isolates expression of AfuA. (A) Agarose gels showing amplicons from PCRs performed on clinical isolates (lanes 1–14) or BP536 (lane 15) with primers for *afuA*. PCR negative control was performed in parallel (lane 16). (B) Cell lysate of clinical isolates (lanes 1, 2, 3, 4, and 5), and BP536 (lane 6) grown under iron limitation were subjected to SDS-PAGE (10%) and immunoblot analysis using anti-rAfuA antibodies. The gel was loaded with the same amount of proteins of each sample.

concentrations of $MgSO_4$ showed that AfuA is expressed both in Bvg+ and Bvg− *B. pertussis*. Bvg− mutant (BP537) was included as a control (Fig. 5). Western blot analysis of Bvg+ and Bvg− *B. pertussis* using 37F3 (IgG1) monoclonal antibody against *B. pertussis* FHA (kindly provided by The Netherlands Vaccine Institute, Bilthoven, The Netherlands) were ran in parallel to confirm bacterial modulation induced by the presence of $MgSO_4$ in the culture media (data not shown).

3.6. rAfuA protects mice against infection

Mice vaccinated with rAfuA/FA were challenged intranasally with iron restricted virulent BP536. Three days post infection mice were sacrificed and the number of CFU/lung was determined. Bacterial clearance in the lungs was used to assess protective activity. Mice immunized with the recombinant protein plus adjuvant were significantly ($P < 0.05$) more protected against *B. pertussis* than the mice immunized with adjuvant alone (Table 1). This level of protection persisted on day 8 (Table 1).

3.7. rAfuA and/or rIRP1-3 improves the protection conferred by a DTPa vaccine

We next investigated the effect of the addition of rAfuA and rIRP1-3, another iron repressed protein that proved protective against pertussis infection [20], compared to a three-pertussis-components DTPa vaccine. Both rAfuA and rIRP1-3 either alone or in combination significantly ($P < 0.05$) enhanced the level of protection of the DTPa vaccine. Moreover, the five-pertussis-components DTPa-rIRP1-3-rAfuA vaccine exhibited a significantly higher protection against *B. pertussis* than DTPa, DTPa + rIRP1-3, or DTPa + rAfuA ($P < 0.05$) (Table 1).

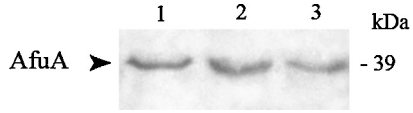

Fig. 5. AfuA Bvg-regulation. Immunoblot analysis of BP536 grown in iron restricted media in the presence (lane 1) or the absence (lane 2) of 40 mM of MgSO4, and BP537 (Bvg−) grown in iron restricted media (lane 3). Whole cell lysates were subjected to SDS-PAGE (10%) and immunoblot analysis using anti-rAfuA antibodies.

Table 1
Protection against *B. pertussis* infection induced by rAfuA immunization and contribution of rAfuA and rIRP1-3 to the protection conferred by DTPa.

Vaccine	Day 3 Units of protection[a] (Log CFU in lungs[b])	Day 8 Units of protection[a] (Log CFU in lungs[b])
rAfuA/FA	0.92 (6.05 ± 0.52[c,d])	1.15 (5.69 ± 0.45[c,d])
DTPa + rAfuA	3.82 (3.07 ± 0.36[c,d])	4.01 (3.01 ± 0.34[c,d])
DTPa + rIRP1-3	3.96 (2.93 ± 0.53[c,d])	4.05 (2.97 ± 0.50[c,d])
DTPa + rAfuA + rIRP1-3	4.15 (2.74 ± 0.38[c,d,e])	4.31 (2.71 ± 0.46[c,d,e])
DTPa	3.30 (3.59 ± 0.38[c])	3.43 (3.59 ± 0.45[c])
PBS/FA	– (6.97 ± 0.46)	– (6.84 ± 0.33)
PBS/Alum	– (6.89 ± 0.30)	– (7.02 ± 0.43)

[a] Units of protection were obtained by subtraction the mean log CFU of the vaccinated group from the mean log CFU of their respective control immunized group.
[b] The content of bacteria in lungs is represented as the mean log CFU ± SD per group.
[c] Significantly different from the respective control group $P < 0.05$.
[d] Significantly different from DTPa immunized mice $P < 0.05$.
[e] Significantly different from DTPa + rIRP1-3 and DTPa + rAfuA immunized mice $P < 0.05$.

4. Discussion

Whole cell vaccines against pertussis introduced in the 1940s to 1960s, were replaced by more defined and less reactogenic acellular pertussis vaccines in the 1990s [31] consisting of detoxified virulence factors derived from *B. pertussis*. Despite universal vaccination, this disease continued to be a major public health problem. Pertussis is nowadays one of the worst controlled vaccine-preventable diseases, whose incidence is increasing despite massive vaccination [32,33]. The reemergence of pertussis has been attributed to pathogen adaptation and suboptimal vaccines, among others factors. Antigenic divergence between vaccine strains and clinical isolates has been extensively reported [34,35]. In addition to vaccine components antigenic variation, the recently reported existence of circulating strains that are deficient in some of the antigens included in the current Pa vaccines, like PT and Prn [9–11] demonstrate that vaccines need to be revised. Several studies have been devoted to the development of improved vaccines. New proposed vaccine antigens include *Bordetella* colonization factor A (BcfA) [36] and autotransporters like BrKA [37]. Other studies are focused on developing mutant strains defective in toxic components to be used as live attenuated vaccines [38,39]. Our approach is based on the hypothesis that the efficacy of existing Pa vaccines may be improved by addition of further *B. pertussis* antigens, such as those whose expression is induced during and required for host colonization. There are many studies that support the assumption that *B. pertussis* is iron-starved and able to use different sources of iron during infection [15–18]. This pathogen is transmitted from host to host so infecting bacteria is expected to exhibit an iron starved phenotype. In search of new vaccine components we focused on outer membrane associated proteins regulated by the environmental iron level. By mean of comparative proteomics combined with serological proteome analysis we identified two putative iron binding proteins in the outer membrane subproteome of iron starved *B. pertussis*, over-expressed under this environmental condition and differentially recognized by antibodies induced by infection [19]. One of these proteins, IRP1-3, was recently found highly immunogenic and protective against *B. pertussis* infection in mouse model [20]. In the present study we evaluated the immunogenicity and protective capacity of the other protein, BP1605, designated AfuA, and further examined the protective efficacy of these two recombinant proteins, rAfuA and rIRP1-3, in combination with the commercial three-pertussis-component vaccine, *Infanrix*™.

Active immunization of mice with rAfuA resulted in the induction of high titers of IgG anti-rAfuA antibodies. Protection against *B. pertussis* is mediated by both humoral and cell-mediated immunity and evidence suggests that cell-mediated immunity is critical for protection [40]. The analysis of IgG isotypes induced by rAfuA showed a balanced IgG1/IgG2a antibody response. Given that IgG1 and IgG2a are known to reflect the stimulation of Th2 and Th1 cells, respectively, the induction of specific antibodies of both subclasses suggest the stimulation of both Th1 and Th2 type of immune responses.

AfuA of *B. pertussis* has significant homology with other ferric binding proteins, such as FbpA from *Neisseria meningitides*, whose cellular location remain controversial [41–44]. Recently, Brickman et al. found AfuA among the proteins released by osmotic shock [25] which led them to suggest that AfuA of *B. pertussis* is a periplasmic protein. Our results show that anti-AfuA specific antibodies recognize the protein on the surface of iron starved bacteria suggesting that AfuA, or at least part of it, is exposed on the bacterial surface. Surface proteins of *B. pertussis* are attractive vaccine antigens particularly if they are able to induce opsonic antibodies, critical for cell mediated bacterial clearance [27,45,46]. Among the antigens included in Pa vaccines, solely Prn, a highly polymorphic antigen [47], induces antibodies with opsonic activity [48]. The recent finding of Prn-negative *B. pertussis* among circulating bacteria [9–11] highlights the importance of identifying new opsonin targets to improve current vaccines. We here showed that anti-rAfuA antibodies opsonize *B. pertussis*, and promote efficient bacterial phagocytosis by human PMN. Accordingly, immunization of mice with rAfuA/FA resulted in a lowering of lung bacterial burdens even in monovalent formulation. Interestingly, although this protection might be due to the opsonic activity of anti-AfuA antibodies, antibodies with this specificity might also be protective in other ways, like obstructing iron uptake systems that were shown critical for *in vivo* survival [25].

According to our results, like IRP1-3 [20], AfuA is a non-BvgAS regulated protein. All the antigens included in the current acellular vaccines and those which were proposed as new antigens, like BrkA and BcfA, are virulent factors positively regulated by BvgAS system. There are studies indicating that modulating conditions in the intranasal cavity [49] might eventually induce the temporal lack of expression of one or more of these vaccine antigens in the infecting bacteria. Thus, the addition of new antigens expressed irrespectively of the virulence state of the bacteria, like AfuA and IRP1-3, might help to limit the first states of the infection.

Sequence conservation among strains and expression during human infection are characteristics that a vaccine candidate has to meet. This study shows that *afuA* gene is highly conserved in clinical isolates and it is expressed under iron starvation. Importantly, sera from infected individuals showed high anti-rAfuA antibody titers confirming that the protein is expressed during infection and immunogenic in humans.

Finally, given the protective activity of rAfuA and rIRP1-3 in monovalent formulations we investigated the effect of the addition of these antigens on the level of protection conferred by an acellular vaccine currently in use. Our results showed that either rAfuA or rIRP1-3 significantly increased the protective capacity of DTPa vaccine suggesting a potential additive effect of IRP1-3 and AfuA on the protection conferred by vaccination.

In summary, the data presented in this study show that both AfuA and IRP1-3 are promising candidates to improve current Pa vaccines.

Acknowledgments

The authors thank the Laboratorio Central, Sala de Microbiología, Hospital de Niños Superiora Sor María Ludovica, La Plata, Argentina for providing the clinical *B. pertussis* isolates. This study was partially supported the ANPCyT (PICT 0559). M.E.R., Y.L., and

G.P. are members of the Scientific Career of CONICET. E.E. and J.A.H. are fellows of CONICET.

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