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Natural-host animal models indicate functional interchangeability between the filamentous haemagglutinins of *Bordetella pertussis* and *Bordetella bronchiseptica* and reveal a role for the mature C-terminal domain, but not the RGD motif, during infection

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Summary

Bacteria of the *Bordetella* genus cause respiratory tract infections. Both broad host range (e.g. *Bordetella bronchiseptica*) and human-adapted (e.g. *Bordetella pertussis*) strains produce a surface-exposed and secreted protein called filamentous haemagglutinin (FHA) that functions in adherence and immunomodulation. Previous studies using *B. pertussis* and cultured mammalian cells identified several FHA domains with potential roles in host cell interactions, including an Arg-Gly-Asp (RGD) triplet that was reported to bind integrins on epithelial cells and monocytes to activate host signalling pathways. We show here that, in contrast to our previous report, the *fhaB* genes of *B. pertussis* and *B. bronchiseptica* are functionally interchangeable, at least with regard to the various *in vitro* and *in vivo* assays investigated. This result is significant because it indicates that information obtained studying FHA using *B. bronchiseptica* and natural-host animal models should apply to *B. pertussis* FHA as well. We also show that the C-terminus of mature FHA, which we name the MCD, mediates adherence to epithelial and macrophage-like cells and is required for colonization of the rat respiratory tract and modulation of the inflammatory response in mouse lungs. We could not, however, detect a role for the RGD in any of these processes.

Introduction

Pertussis, or whooping cough, is an acute respiratory disease that is increasing in incidence despite widespread vaccine coverage (Deville *et al.*, 1995; Campos-Outcalt, 2005; Wood and McIntyre, 2008). The causative agents, *Bordetella pertussis* and *Bordetella*

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parapertussis$_{hu}$, are Gram-negative bacteria that infect only humans. Phylogenetic analyses indicate that these bacteria diverged independently and relatively recently from *Bordetella bronchiseptica* or a *B. bronchiseptica*-like ancestor and that all three 'species' are so closely related that they should be considered subspecies or strains of the same species (Arico *et al.*, 1987; Bemis, 1992; van der Zee *et al.*, 1997; Parkhill *et al.*, 2003; Cummings *et al.*, 2004; Diavatopoulos *et al.*, 2005). Despite this remarkable similarity, *B. bronchiseptica* differs significantly from *B. pertussis* and *B. parapertussis*$_{hu}$ by displaying a broad host range that includes animals commonly studied in the laboratory such as rabbits, rats, guinea pigs and mice (Bemis, 1992).

Filamentous haemagglutinin (FHA) was one of the first *B. pertussis* virulence factors to be discovered (Arai and Sato, 1976; Sato *et al.*, 1981). It is a large, rod-shaped, highly immunogenic protein that is both surface-associated and secreted and is a primary component of acellular pertussis vaccines (Arai and Sato, 1976; Sato *et al.*, 1981; Jacob-Dubuisson *et al.*, 1999; 2000; Sato and Sato, 1999). A prototypical member of the Two Partner Secretion (TPS) pathway family, FHA is first synthesized as an ~370 kDa preproprotein called FhaB that loses its 71-amino-acid (aa) signal sequence during Sec-dependent secretion across the cytoplasmic membrane (Jacob-Dubuisson *et al.*, 1996; Chevalier *et al.*, 2004). The N-terminal ~250 aa 'TPS domain' targets the proprotein in the periplasm to FhaC, an outer membrane, β-barrel, channel-forming protein that is required for translocation of FhaB to the cell surface (Clantin *et al.*, 2004; 2007; Hodak *et al.*, 2006; Meli *et al.*, 2006). At some point during translocation, FhaB is cleaved in an SphB1-dependent manner to form the mature ~240 kDa FHA protein (Coutte *et al.*, 2001; Mazar and Cotter, 2006). SphB1-dependent cleavage occurs in at least two locations, one somewhere between aa 2362 and 2372 (based on the aa numbering of FhaB predicted from the *fhaB* gene of *B. pertussis* Tohama 1) and the other approximately 100 aa N-terminal to that site (Coutte *et al.*, 2001; Mazar and Cotter, 2006). The importance of cleavage at one site versus the other is unknown. The ~130 kDa C-terminal prodomain that is released upon cleavage cannot be detected in whole cell lysates (WCLs) or concentrated supernatants, presumably due to its rapid degradation (Renauld-Mongenie *et al.*, 1996; Mazar and Cotter, 2006). X-ray crystallography, high-resolution electron microscopy, and modelling studies indicate that mature FHA is shaped like a horseshoe nail, with the N-terminal ~2000 aa forming a β-helix that makes up the 'shaft' and the C-terminal ~500 aa forming a globular domain (which we call the MCD, for mature C-terminal domain) at one end (Kajava *et al.*, 2001; Clantin *et al.*, 2004). We showed recently that mature cell-associated FHA is oriented such that its C-terminus (the MCD), and not its N-terminus, is located distally from the cell surface and is accessible to antibodies (Mazar and Cotter, 2006).

In vitro studies using *B. pertussis* or FHA purified from *B. pertussis* have identified three putative functional domains (see Fig. 3 for a schematic showing their relative locations). A heparin binding domain (HBD) located near the N-terminus of FHA has been reported to mediate attachment to sulphated polysaccharides (Hannah *et al.*, 1994). A carbohydrate recognition domain (CRD) located near the centre of the protein has been reported to mediate adherence to respiratory epithelial cells and macrophages (Prasad *et al.*, 1993) and an arg-gly asp (RGD) triplet, located just N-terminal to the CRD, has been reported to interact with the leucocyte response integrin/ integrin-associated protein (LRI/IAP) complex on monocytes/macrophages, resulting in upregulation of complement receptor 3 (CR3) binding activity (Ishibashi *et al.*, 1994), and with very late antigen 5 (VLA-5) on epithelial cells to stimulate the upregulation of intercellular adhesion molecule 1 (ICAM-1) (Ishibashi and Nishikawa, 2002; 2003). FHA has also been reported to inhibit antigen-dependent $CD4^+$ T cell proliferation and to induce apoptosis when incubated with monocytes/macrophages (Boschwitz *et al.*, 1997a; Abramson *et al.*, 2001) and to induce immunosuppressive effects on murine macrophages and dendritic cells by downregulating production of IL-12 in an

IL-10-dependent manner (McGuirk and Mills, 2000; McGuirk *et al.*, 2002). Based on these and other studies, FHA has been proposed to function as an adhesin and an immunomodulator.

Studies aimed at determining roles for FHA *in vivo* using *B. pertussis* and mouse models have yielded conflicting data, with most failing to reveal any difference between wild-type and FHA-deficient bacteria (Weiss and Goodwin, 1989; Goodwin and Weiss, 1990; Kimura *et al.*, 1990; Roberts *et al.*, 1993; Khelef *et al.*, 1994; Alonso *et al.*, 2001; McGuirk *et al.*, 2002). Lack of a clear phenotype for *fhaB* mutants in these studies may be due to the fact that mice are not natural-hosts for *B. pertussis*.

The FhaB protein produced by *B. bronchiseptica* strain RB50 is predicted to be 90% identical and 93% similar to FhaB of *B. pertussis* strain Tohama 1 (Parkhill *et al.*, 2003) and *in vitro* studies have shown FHA to be both necessary and sufficient for mediating adherence of *B. bronchiseptica* to a variety of epithelial and macrophage cell lines (Cotter *et al.*, 1998; Mattoo *et al.*, 2000; Inatsuka *et al.*, 2005; Julio and Cotter, 2005). By contrast with studies using *B. pertussis*, animal experiments revealed dramatic differences between wild-type and FHA-deficient *B. bronchiseptica*. For example, while wild-type *B. bronchiseptica* persistently colonizes both the nasal cavity and trachea of rats and mice inoculated with a relatively small number of bacteria delivered in a small volume to the nares, Δ*fhaB* mutants are only able to colonize the nasal cavity, and often with decreased efficiency (Cotter *et al.*, 1998; Mattoo *et al.*, 2000; Julio and Cotter, 2005). Mice inoculated with a large number of FHA-deficient *B. bronchiseptica* in a large volume that deposits bacteria into the lungs produce a robust inflammatory response that is often fatal while those inoculated with the same number of wild-type bacteria remain healthy (Inatsuka *et al.*, 2005). These data suggest that FHA contributes to colonization of the lower respiratory tract by allowing *B. bronchiseptica* to suppress the inflammatory response. We reported previously that the *fhaB* gene of *B. pertussis* ($fhaB_{Bp}$) could not substitute for the *fhaB* gene of *B. bronchiseptica* ($fhaB_{Bb}$) during infection (Inatsuka *et al.*, 2005). The goals of the current study were to determine the molecular basis for the lack of $fhaB_{Bp}$ and $fhaB_{Bb}$ interchangeability *in vivo* and to investigate the roles of the RGD triplet and the C-terminus of the mature FHA protein (the MCD) in pathogenesis.

Results

Construction and in vitro characterization of a *B. pertussis* Tohama 1 derivative expressing *fhaB* from *B. bronchiseptica* RB50

We reported previously that $fhaB_{Bp}$ could substitute for $fhaB_{Bb}$ *in vitro* but not *in vivo* (Inatsuka *et al.*, 2005). To determine if $fhaB_{Bb}$ could substitute for $fhaB_{Bp}$, we constructed a *B. pertussis* strain expressing $fhaB_{Bb}$. Bp536 is a streptomycin-resistant derivative of Tohama 1, the *B. pertussis* strain for which the genome sequence was determined (Parkhill *et al.*, 2003). We used allelic exchange to construct a derivative of Bp536, called Bpe138, in which the entire $fhaB_{Bp}$ coding sequence was replaced with a gene encoding chloramphenicol resistance (Fig. 1A). Plasmid pSJ63 contains all but the first 70 codons of $fhaB_{Bb}$ from RB50 fused to the $fhaB_{Bp}$ promoter region plus the first 70 codons of $fhaB_{Bp}$. The first 70 aa encoded by $fhaB_{Bp}$ and $fhaB_{Bb}$ differ at only two positions and because the FhaB signal sequence is 71 aa long, neither of those aa are present in the FhaB proprotein or mature FHA. Integration of pSJ63 into the chromosome of Bpe138 via recombination within the promoter region (confirmed by PCR) resulted in a *B. pertussis* strain (Bpe138::pSJ63) that expressed $fhaB_{Bb}$ (with the first 70 codons from $fhaB_{Bp}$) from the native *B. pertussis* *fhaB* promoter. Expression of genes 3′ to *fhaB* (*fimBCD* and *fhaC*) is expected to be the same in this strain as in Bp536 and Bpe138. [Note that *fimA* in *B. pertussis* (*'fimA*) lacks

transcription and translation initiation sequences and the *fimBCDfhaC* operon is transcribed from the *fimB* promoter (Boschwitz *et al.*, 1997b).]

Western blot analysis showed that Bpe138 did not produce FHA and that Bpe138::pSJ63 produced and secreted FHA_{Bb}, which is slightly larger than FHA_{Bp} (Fig. 1B). [The fact that FHA can be detected in strain Bpe138::pSJ63 provides functional evidence that genes downstream of *fhaB* are expressed because neither FhaB nor FHA can be detected in *fhaC* mutants (Willems *et al.*, 1994; Jacob-Dubuisson *et al.*, 1997; Julio and Cotter, 2005).] As shown previously, maturation of the ~370 kDa FhaB proprotein to mature ~250 kDa FHA appears to be more efficient in *B. pertussis* Tohama 1 and its derivatives than in *B. bronchiseptica* RB50 and its derivatives because the ~370 kDa FhaB proprotein is visible in WCLs of RB50 but not Tohama 1 derivatives such as Bp536 (Fig. 1B) and BPSM (Mazar and Cotter, 2006). Also as shown previously, Tohama 1 derivatives release more FHA into culture supernatants than RB50 and its derivatives (Fig. 1B and Mazar and Cotter, 2006). Both strain background and the specific aa sequences of the FhaB proteins apparently contribute to both phenotypes because the amount of the ~370 kDa FhaB proprotein visible in WCLs of Bpe138::pSJ63 and the amount of FHA visible in culture supernatants of Bpe138::pSJ63 are intermediate between those of Bp536 and RB50 (note that the supernatant sample used for Bp536 was diluted twofold compared with the others) (Fig. 1B).

We and others have shown that FHA contributes to adherence of *B. pertussis* to epithelial and macrophage-like cell lines *in vitro* (Relman *et al.*, 1990; Inatsuka *et al.*, 2005). Consistent with these previous data, Bpe138 was less able to adhere to rat lung epithelial (L2) cells than Bp536 (Fig. 1C). Expression of $fhaB_{Bb}$ in Bpe138 restored its ability to adhere to L2 cells (Fig. 1C) and J774A.1 macrophage-like cells (data not shown). The FHA_{Bb} protein produced in Bpe138::pSJ63 is therefore functional with regard to mediating adherence to cultured cells.

Substitution of *fhaB* in *B. pertussis* Tohama 1 with *fhaB* from *B. bronchiseptica* RB50 does not alter the ability of *B. pertussis* Tohama 1 to cause respiratory infection in rats or mice

Inoculation of Wistar rats with as few as 20 colony-forming units (cfu) of *B. bronchiseptica* results in colonization of the nasal cavities and tracheas with high numbers of bacteria by day 10 post inoculation (Akerley *et al.*, 1995). *B. bronchiseptica fhaB* mutants are unable to colonize the rat trachea and show decreased ability to colonize the nasal cavity (Cotter *et al.*, 1998; Mattoo *et al.*, 2000; Inatsuka *et al.*, 2005; Julio and Cotter, 2005). Wild-type *B. pertussis*, by contrast, is unable to colonize either the nasal cavity or trachea of rats, even when inoculated at a dose of 10^6 cfu (our unpublished observation). To determine if FHA_{Bb} could increase the ability of *B. pertussis* to establish respiratory infection in rats, we inoculated Wistar rats intranasally with 1×10^6 cfu of Bp536, Bpe138 and Bpe138::pSJ63. No *Bordetella* were recovered from the nasal cavities or tracheas of any *B. pertussis*-inoculated animal at 14 days post inoculation (data not shown). As a control to demonstrate that the $fhaB_{Bb}$ gene contained on plasmid pSJ63 encodes an FHA protein that is functional *in vivo*, we also inoculated rats with *B. bronchiseptica* strains RBX11, RBX20 and RBX20::pSJ61. RBX11 is an RB50 derivative containing a large in-frame deletion mutation in *fhaS*. *fhaS* is an *fhaB* homologue that plays no discernible role in the infection of rats or mice by *B. bronchiseptica* (Julio and Cotter, 2005), but which, due to its high degree of nucleotide identity with *fhaB*, complicates genetic manipulation of the *fhaB* gene. RBX20 is an RB50 derivative containing large in-frame deletion mutations in *fhaB* and *fhaS*. pSJ61 is the parent plasmid of pSJ63. It contains the entire *fhaB* gene and promoter region from RB50 (Fig. 1A). At day 14 post inoculation with 1000 cfu, RBX11 and RBX20::pSJ61 were recovered from the nasal cavities and tracheas at high numbers while RBX20 was not recovered from any trachea and was recovered at low numbers from the nasal cavities of inoculated animals (data not shown). Together, these results indicate that the *fhaB* gene on

pSJ61 (and hence pSJ63) encodes an FHA protein that is functional *in vivo*, but that production of this protein is not sufficient to allow *B. pertussis* to establish respiratory infection in rats.

Although it does not reflect a natural course of infection, inoculation of mice intranasally with a large number of bacteria (5×10^5 cfu) delivered in a large volume (50 μl) has proven to be a useful model for investigating the ability of *Bordetella* to induce an inflammatory response and to resist clearance by inflammatory cells (Harvill *et al.*, 1999a,b; Inatsuka *et al.*, 2005). Consistent with our previously published results (Inatsuka *et al.*, 2005), ~1×10^6 cfu of RB50 were recovered from the lungs at day 4 post inoculation and ~1×10^5 cfu of RB50 were recovered at day 11 post inoculation using this protocol (Fig. 2A). Also consistent with our previous results, inoculation with the Δ*fhaB* strain, RBX20, resulted in a bimodal response; half of the animals became moribund by day 4 and very high numbers (~1×10^9) of cfu were recovered from their lungs and half of the animals remained healthy and the number of cfu recovered from their lungs was slightly lower than the number recovered from the lungs of RB50-inoculated animals (Fig. 2A). For RBX20 therefore the LD_{50} is ~5×10^5 cfu when inoculated using this protocol. In animals that remained healthy, the number of cfu recovered from the lungs at day 11 post inoculation was very low. The lungs of animals that appeared healthy at day 4 post inoculation showed only mild inflammation as assessed by microscopic examination of haematoxylin and eosin (H&E)-stained tissue sections, while the lungs of moribund animals showed massive infiltration of inflammatory cells that included primarily neutrophils and lymphocytes and areas of infarction and fluid accumulation were also observed (data not shown and Inatsuka *et al.*, 2005). We have interpreted these data to indicate that FHA functions to modulate the robustness of the inflammatory response; without FHA, a (hyper)inflammatory response is induced that either clears the bacteria quickly, or causes local tissue damage that promotes increased bacterial growth, more inflammation, more damage and ultimately death of the mouse (Inatsuka *et al.*, 2005). RBX20::pSJ61 was recovered from the lungs at numbers similar to RB50 at all time points (Fig. 2A) demonstrating that the $fhaB_{Bb}$ gene contained on pSJ61 (and pSJ63) encodes a protein that is capable of allowing *B. bronchiseptica* to modulate the inflammatory response in mice. When inoculated using this protocol, the number of cfu of *B. pertussis* recovered from the lungs of mice also increases at days 3–7 post inoculation, then decreases over time, but differences between wild-type and *fhaB* mutant strains have not been consistently observed and the bimodal response that we observed for the Δ*fhaB* strain of *B. bronchiseptica* has not been reported for *fhaB* mutant strains of *B. pertussis* (Kimura *et al.*, 1990; Khelef *et al.*, 1994; Harvill *et al.*, 1999a; 2000; Alonso *et al.*, 2001). To determine if $fhaB_{Bb}$ alters the interaction between *B. pertussis* and the murine respiratory tract, we inoculated mice with our various *B. pertussis* strains. Consistent with previous reports (Kimura *et al.*, 1990; Khelef *et al.*, 1994; Harvill *et al.*, 1999a; 2000; Alonso *et al.*, 2001), ~3×10^6 and ~2×10^5 cfu of Bp536 were recovered from the lungs at days 4 and 11 post inoculation respectively (Fig. 2A). Also consistent with previous reports (Kimura *et al.*, 1990; Khelef *et al.*, 1994; Alonso *et al.*, 2001), the number of cfu of the Δ*fhaB B. pertussis* strain (Bpe138) recovered from the lungs was not significantly different from the number of cfu of its wild-type parental strain (Bp536) recovered at any time point. Moreover, examination of H&E-stained lung sections revealed only mild inflammation in all *B. pertussis*-inoculated animals (data not shown). In contrast to the case with *B. bronchiseptica* therefore, there was no evidence that a more robust inflammatory response was induced in animals inoculated with Δ*fhaB B. pertussis* strains compared with wild-type *B. pertussis*. The number of cfu recovered from the lungs of Bpe138::pSJ61-inoculated animals at all time points was not different from those recovered from Bp536- and Bpe138-inoculated animals (Fig. 2A).

As an additional assessment of the ability of the various strains to establish infection, we compared the serum antibody responses of inoculated animals by Western blot analysis. As shown previously (Harvill *et al.*, 1999a), sera from RB50-inoculated mice contain antibodies that recognize *Bordetella*-specific antigens, such as lipopolysaccharide, adenylate cyclase and FHA (Fig. 2B). Also consistent with our previous results (Inatsuka *et al.*, 2005), the antibody response generated in animals inoculated with FHA-deficient *B. bronchiseptica* is weak. The Western blot results obtained using sera from Bp536- and Bpe138::pSJ63-inoculated animals did not differ from those obtained using sera from phosphate-buffered saline (PBS)-inoculated animals (Fig. 2B). These results indicate, consistent with previous results (Harvill *et al.*, 1999a), that inoculation of mice with *B. pertussis* does not result in an antibody response that can be detected by Western blot, and that although expression of FHA_{Bb} contributes to the ability of *B. bronchiseptica* to induce a strong antibody response, it does not alter the antibody response induced by *B. pertussis*.

Taken together, these results indicate that expression of $fhaB_{Bb}$ in *B. pertussis* does not alter its ability to establish respiratory infection in rats, to induce an inflammatory response in mice, to resist clearance by inflammatory cells in mice, or to induce adaptive immunity in mice. FHA_{Bb} therefore does not improve the ability of *B. pertussis* to infect rats and mice. Whether FHA_{Bb} can actually substitute for FHA_{Bp} *in vivo*, however, cannot be concluded from these experiments because these *in vivo* assays, which use an animal not normally infected by *B. pertussis*, could not distinguish Δ*fhaB B. pertussis* from wild-type *B. pertussis.*

FHA_{Bp} can substitute for FHA_{Bb} with regard to rat and mouse infection

We reported previously that FHA_{Bp} could not substitute for FHA_{Bb} in *B. bronchiseptica in vivo* (Inatsuka *et al.*, 2005). The strain used in that study, RBFS4, was believed to contain and express an intact wild-type *fhaB* allele from *B. pertussis*: it produced FHA_{Bp} of the expected mature size that was localized on the surface of the bacteria and was also secreted in a manner similar to wild-type *B. bronchiseptica*. Like RB50, RBFS4 was able to adhere to epithelial cells and macrophages (albeit with somewhat reduced ability), but like RBX9 (the *B. bronchiseptica* Δ*fhaB* derivative), RBFS4 was unable to colonize the tracheas of rats and it elicited a hyperinflammatory response in mouse lungs. Because a majority of the aa differences between FHA_{Bb} and FHA_{Bp} are within the HBD, the CRD and the N-terminal half of the MCD, we constructed *B. bronchiseptica* strains producing chimeric FHA proteins (Fig. 3) to determine if differences in one of these regions could account for the different phenotypes displayed by RB50 and RBFS4. All of these strains were indistinguishable from RB50 in their ability to adhere to cultured cells and to infect rats and mice (data summarized in Fig. 3). At the same time that we were conducting these experiments, we found that deletions in the region of *fhaB* encoding the C-terminal prodomain (which is removed from the mature FHA protein at some point in the secretion process and which cannot be detected as a separate protein in WCLs or culture supernatants) resulted in strains that produced and secreted mature FHA but were unable to colonize the tracheas of rats (Mazar and Cotter, 2006). One of these strains (RBX11-T-E) was able to adhere to epithelial and macrophage-like cell lines, but with slightly reduced ability compared with RB50 (Fig. 3 and Mazar and Cotter, 2006). This strain therefore displayed a phenotypic profile identical to that of RBFS4 (although the inflammatory response to RBX11-T-E was not investigated in the previous study). In light of this information, and the fact that expression of $fhaB_{Bb}$ in *B. pertussis* did not alter its ability to infect rats or mice, we resequenced the DNA region in RBFS4 encoding the prodomain. This new sequence information revealed three thymidines instead of two at nucleotide position 7126–7127 (relative to the adenosine in the translational start codon of $fhaB_{Bb}$), which is located about 10 codons 3′ to the region encoding the putative primary SphB1-dependent maturation site. The additional thymidine is predicted to shift the

reading frame such that it is followed by 42-aa-encoding codons and then a stop codon. Using allelic exchange, we 'repaired' the frame-shift mutation in RBFS4 to create RBFS10. RBFS10 was indistinguishable from RB50 in its ability to adhere to epithelial and macrophage-like cells, to colonize the tracheas of rats, and to colonize the lungs of mice with the induction of only a mild inflammatory response (Fig. 3). These results show that, in contrast to our previously published report, the *fhaB* gene from *B. pertussis* can substitute for that of *B. bronchiseptica* with regard to these *in vitro* and *in vivo* phenotypes. These data also show that individual FHA domains are also interchangeable, not just the entire molecules.

The FHA RGD triplet does not appear to play a role in the ability of *B. bronchiseptica* RB50 to cause respiratory infection in rats and mice

The results described above indicate that FHA_{Bb} and FHA_{Bp} are functionally interchangeable, at least with regard to the *in vitro* and *in vivo* phenotypes that we have studied. Information gleaned about FHA function from studies using *B. bronchiseptica* may therefore apply to FHA function in *B. pertussis* as well. Several reports using *B. pertussis,* FHA purified from *B. pertussis*, and various cell culture models have suggested a role for the RGD triplet located near the middle of the mature FHA protein in pathogenesis (Relman *et al.*, 1990; Ishibashi *et al.*, 1994; 2001; 2002; Ishibashi and Nishikawa, 2002; 2003). To investigate the role of the RGD triplet *in vivo*, we constructed *B. bronchiseptica* strains producing FHA proteins in which the RGD was replaced with RAD, RGE or RAE. These substitutions were chosen because although they are relatively conservative changes, each individually has been shown to abrogate cell-attachment activity of RGD containing proteins (Pierschbacher and Ruoslahti, 1984). All of the strains produced and secreted FHA proteins that were indistinguishable in size and amount from RB50 and all of the strains adhered to L2 cells and J774A.1 cells in a manner indistinguishable from RB50 (data not shown). We hypothesized that the RAE substitution, which differed the most from the wild-type sequence, would be the most likely to alter FHA function and therefore we used only the strain expressing this FHA mutant (which we called RB50RAE) in all subsequent experiments. RB50RAE was indistinguishable from RB50 in its ability to colonize the nasal cavities and tracheas of rats (Fig. 4A) and it was recovered at the same numbers as RB50 from the lungs of mice at days 3 and 11 post inoculation (Fig. 4B). Lung tissues from mice infected with RB50 and RB50RAE that were sectioned and stained with H&E were indistinguishable (data not shown). RB50RAE was also similar to RB50 in its ability to cause a lethal infection in immunodeficient SCID/Bg mice following both low-dose and high-dose inoculation (Fig. 4C and D). These results indicate that the RGD motif does not contribute to the ability of *B. bronchiseptica* to establish respiratory infections in rats, to induce or suppress an inflammatory response in mice, or to resist inflammation-mediated clearance in mice, at least not in an way that can be distinguished using this repertoire of animal models.

The FHA RGD triplet does not appear to contribute to *Bordetella*-induced ICAM-1 surface expression in respiratory epithelial cells

Lack of a difference between RB50 and RB50RAE in rats and mice was somewhat unexpected given previous publications (Relman *et al.*, 1990; Ishibashi *et al.*, 1994; 2001; 2002; Ishibashi and Nishikawa, 2002; 2003). Among the activities attributed to the FHA RGD is the upregulation of ICAM-1 on the surface of epithelial cells (Ishibashi and Nishikawa, 2002). We therefore determined if *B. bronchiseptica* could induce surface expression of ICAM-1 in BEAS-2B cells, and, if so, if FHA, and specifically the RGD triplet of FHA, was required for this ability. We incubated BEAS-2B cells with RB50, RBX9, RB50RAE and also the *B. pertussis* strains used in previous studies, at a multiplicity of infection (moi) of 100 and measured surface ICAM-1 by flow cytometry. Both *B.*

bronchiseptica and *B. pertussis* caused a modest increase in surface expression of ICAM-1 in an FHA-dependent manner, consistent with previous studies with *B. pertussis* (Fig. 5) (Ishibashi and Nishikawa, 2002). By contrast with those studies, however, the RGD motif was not required for this activity because the amount of ICAM-1 on the surface of BEAS-2B cells following incubation with the *B. pertussis* RAD mutant and the *B. bronchiseptica* RAE mutant was the same as following incubation with either wild-type strain (Fig. 5). These data indicate that the FHA RGD motif does not contribute to the increased surface expression of ICAM-1 that occurs in epithelial cells in response to exposure to either *B. bronchiseptica* or *B. pertussis*.

Antibodies against the MCD, but not those against the CRD, block FHA-mediated adherence of *B. pertussis* and *B. bronchiseptica* to epithelial and macrophage-like cell lines

We showed recently that the C-terminus of mature FHA (the MCD) is exposed distally on the cell surface (Mazar and Cotter, 2006). The CRD and the RGD, however, have been suggested to mediate interactions with host cells (Relman *et al.*, 1990; Prasad *et al.*, 1993; Ishibashi *et al.*, 1994; 2002; Ishibashi and Nishikawa, 2002; 2003). To investigate the importance of these regions in adherence, we incubated bacteria with anti-CRD or anti-MCD antibodies, or buffer alone, washed away unbound antibody, and then determined the ability of these bacteria to adhere to L2 cells and J774A.1 macrophage-like cells. Pre-incubation with anti-MCD antibodies resulted in adherence levels of *B. pertussis* and *B. bronchiseptica* that were dramatically lower than adherence in the absence of incubation with antibody (Fig. 6A). By contrast, pre-incubation of bacteria with anti-CRD antibodies had no effect on the level of adherence. (Note that the polypeptide used to generate these antibodies included the RGD triplet.) Aliquots of the same bacteria that were used in the adherence assay were also incubated with fluorophore-conjugated secondary antibodies, washed, spotted onto membranes and examined for fluorescence. Both the anti-CRD and anti-MCD antibodies were detected on FHA^+ but not FHA^- *B. bronchiseptica* and *B. pertussis* (Fig. 6B). These results provide evidence that the MCD mediates adherence to epithelial and macrophage-like cells and that at least portions of the CRD are not required for this activity.

The C-terminal domain of mature FHA is required for FHA function *in vivo*

To investigate the importance of the FHA MCD *in vivo*, we constructed a *B. bronchiseptica* strain containing a deletion mutation in *fhaB* that removed 28 codons corresponding to aa 2429–2456 of the preproprotein (centred approximately 30 codons 5′ to the region encoding the primary FHA maturation site). Western blot analysis showed that FHA was produced in this strain, called RBX11Δ28, in amounts similar to that in wild-type *B. bronchiseptica* (Fig. 7A). RBX11Δ28 was able to adhere to L2 cells (Fig. 7B) and macrophage-like cells (data not shown) *in vitro*, but was defective in its ability to colonize the tracheas of rats (Fig. 7C). This strain also displayed a ‘bimodal phenotype’ in mice (identical to that displayed by the Δ*fhaB* strain) in which half of the mice became moribund and contained very high numbers of bacteria in their lungs at day 3 post inoculation and half of the animals remained healthy and contained similar or slightly lower numbers of bacteria in their lungs than animals infected with wild-type *B. bronchiseptica* at day 3 post inoculation (Fig. 7D). Consistent with previous observations, lung sections of moribund animals showed massive infiltration of inflammatory cells while lung sections from animals that appeared healthy showed only mild inflammation (data not shown). These results indicate that an intact MCD is required for FHA function *in vivo*. RBX11Δ28 was also similar to RBX9 (the Δ*fhaB* strain) in its ability to induce surface expression of ICAM-1 in BEAS-2B cells *in vitro*, providing additional evidence that it is the MCD that is important in FHA-dependent host cell interactions (Fig. 5).

Discussion

We reported previously that FHA_{Bp} could not substitute for FHA_{Bb} *in vivo* based on characterization of RBFS4, a *B. bronchiseptica* strain in which the native $fhaB_{Bb}$ gene was replaced with $fhaB_{Bp}$ (Inatsuka *et al.*, 2005). Replacement of $fhaB_{Bp}$ with $fhaB_{Bb}$ in *B. pertussis*, however, did not alter the ability of *B. pertussis* to infect rats or mice and 'swapping' experiments, including those shown in Fig. 2, failed to identify a domain within mature FHA_{Bp} responsible for the inability of *B. bronchiseptica* strain RBFS4 to colonize the tracheas of rats or to modulate the immune response in the lungs of mice. These results, together with the discovery that the FhaB prodomain is not required for FHA production, maturation or secretion but is required for rat tracheal colonization (Mazar and Cotter, 2006), led us to re-examine the prodomain-encoding region of $fhaB_{Bp}$ in RBFS4. Repeated sequencing efforts through a particularly difficult section of $fhaB_{Bp}$ DNA in RBFS4 ultimately revealed an additional thymidine approximately 10 codons 3′ to the primary SphB1-dependent maturation site. 'Repair' of the mutation resulted in a strain, RBFS10, that is indistinguishable from RB50 in its ability to adhere to epithelial cells and macrophages, to colonize the tracheas of rats, and to modulate the inflammatory response in the lungs of mice, supporting the conclusion that FHA_{Bp} can in fact substitute for FHA_{Bb} in *B. bronchiseptica in vivo*. We regret our failure to find this mutation initially and our reporting of erroneous conclusions based on the characterization of RBFS4. Re-interpretation of the data obtained with RBFS4 in light of this new information, however, provides some insight into FHA secretion, maturation and function.

The RB50 derivatives that revealed a role for the pro-domain in FHA function (characterized in Mazar and Cotter, 2006) produced $FhaB_{Bb}$ proproteins that were 2588 and 3371 aa in length, i.e. they contained at least the N-terminal ~97 aa of the prodomain. The truncated $FhaB_{Bp}$ proprotein produced in RBFS4 contains only the N-terminal 7–15 aa of the prodomain, followed by 42 aa that differ from those of the native protein, yet RBFS4, like the mutants characterized previously, produced an FHA protein that was translocated to the cell surface, processed in an SphB1-dependent manner, and released into the extracellular milieu in a manner indistinguishable from RB50, and was similarly unable to colonize the tracheas of rats (Inatsuka *et al.*, 2005; Mazar and Cotter, 2006). Characterization of RBFS4 therefore shows that even the very N-terminal region of the prodomain is not required for FHA secretion and processing but is required for FHA-mediated rat tracheal colonization. Moreover, characterization of RBFS4 also showed that the FhaB prodomain is required for *B. bronchiseptica* to modulate the inflammatory response in the lungs of mice, which we had not investigated in our previous study. These data, along with those demonstrating the importance of the MCD *in vivo* (discussed below), support the hypothesis that the role of the prodomain is to control secretion-dependent folding of the MCD and that proper folding of the MCD is critical for FHA function. Consistent with this hypothesis, we have recently obtained evidence from native gel electrophoresis and cysteine availability experiments that conformation of the MCD differs in a prodomain-dependent manner (C.R. Noel, J.A. Sexton, J. Mazar and P.A. Cotter, in preparation).

The fact that the 'repaired' strain, RBFS10, was indistinguishable from RB50 *in vitro* (using both human and non-human cell lines) and *in vivo* indicates that FHA_{Bp} and FHA_{Bb} are functionally interchangeable, at least with regard to the phenotypes that we have investigated. The fact that the *B. bronchiseptica* strains producing chimeric FHA proteins were also indistinguishable from RB50 in these assays indicates that specific domains within FHA are also functionally interchangeable, arguing against the possibility that specific domains (at least those investigated in this study) of FHA_{Bb} or FHA_{Bp} require other domains from the same protein (FHA_{Bb} or FHA_{Bp}) to function properly. We cannot

conclude that FHA_{Bb} can substitute for FHA_{Bp} in *B. pertussis* during the natural course of infection because of the limited host range of this species. However, our results suggest that information obtained studying FHA_{Bb} in *B. bronchiseptica* using natural-host animal models may provide insight into the function of FHA_{Bp} in *B. pertussis* during human infection.

Of the FHA domains identified *in vitro*, the RGD triplet has received the most attention. Reported activities include binding to VLA-5 on epithelial cells to cause increased surface expression of ICAM-1 (Ishibashi *et al.*, 1994; Ishibashi and Nishikawa, 2002; 2003) and binding to LRI/IAP complexes on monocytes to cause enhancement of CR3 binding activity (Ishibashi *et al.*, 1994; 2002). Because wild-type and Δ*fhaB* mutant *B. pertussis* strains are indistinguishable in the mouse model, the role of specific FHA domains cannot be investigated in this context. We reasoned, however, that the sensitive animal models available for use with *B. bronchiseptica* would allow us to identify the role(s) of the FHA RGD during infection, and that the results obtained would reflect the function of both FHA_{Bb} and FHA_{Bp}. We were surprised to find that a strain producing an FHA protein containing an RAE triplet instead of RGD was indistinguishable from wild-type *B. bronchiseptica* in its ability to colonize the respiratory tracts of rats, to modulate the inflammatory response in the lungs of mice, and to cause a lethal systemic infection in immunodeficient mice. Evaluation of our *B. bronchiseptica* strains, and re-evaluation of the *B. pertussis* strains used in previous studies, confirmed that FHA is required for a modest but reproducible induction of ICAM-1 surface expression in epithelial cells, but a role for the RGD motif was not apparent from these experiments. Together, our data indicate that if the RGD triplet contributes to FHA function, its contribution is not evident in these models and assays. The RGD motif of aggregation substance of *Enterococcus faecalis* was similarly shown not to be responsible for the interaction of this pathogen with host cells, despite previous suggestive reports to the contrary (Waters *et al.*, 2003). The degree to which RGD motifs in proteins of bacterial pathogens or symbionts contribute to bacterial–host interactions remains unknown.

Despite the fact that the C-terminal ~500 aa of mature FHA (the MCD) has been shown to be immunodominant in inoculated or immunized mice and rabbits (Delisse-Gathoye *et al.*, 1990; Wilson *et al.*, 1998) and, importantly, in humans recovering from pertussis (Leininger *et al.*, 1997; Piatti, 1999), a role for the MCD in FHA function has not been investigated, or even proposed, previously. Failure to recognize the MCD as a potentially important domain likely stemmed from the assumed ‘N-terminus out’ topology of cell-associated FHA. Our recent discovery that the MCD is located distally from the cell surface and is accessible by antibodies prompted us to consider its contribution to FHA function (Mazar and Cotter, 2006). Our current study shows that a strain producing an FHA protein lacking 28 aa near the C-terminus of the MCD is unable to colonize the tracheas of rats and to modulate the inflammatory response in mice; it displays a phenotype in these animals that is identical to Δ*fhaB* strains. This result demonstrates that aa within the MCD are required for FHA function *in vivo*. Whether these aa are required because they interact directly with a host cell receptor(s) or because they are required for the proper conformation of the FHA protein cannot be distinguished from our data. However, it seems unlikely that a deletion of 28 aa near the C-terminus of the MCD, which is proposed to have a globular structure, would affect the conformation of the β-helical shaft portion of the long, rod-shaped FHA molecule. We are therefore focusing our current efforts on the MCD and are conducting experiments to investigate the potential importance of specific aa within the MCD more thoroughly.

The ability to adhere to primary cells or immortalized cell lines is frequently regarded as an indication of bacterial virulence potential. FHA contributes to the ability of *B. pertussis* to adhere to epithelial cells and macrophages and is both necessary and sufficient for *B. bronchiseptica* to adhere to these cell types *in vitro* (Relman *et al.*, 1990; Ishibashi *et al.*,

1994; Cotter *et al.*, 1998; Mattoo *et al.*, 2000; Inatsuka *et al.*, 2005). *B. bronchiseptica* mutants that do not adhere to cells *in vitro* are defective for tracheal colonization and overcoming inflammatory clearance *in vivo* (Cotter *et al.*, 1998; Inatsuka *et al.*, 2005). These observations suggest that the *in vitro* adherence assay may be a good predictor of FHA function *in vivo* and could potentially be used to investigate the molecular interactions in which FHA participates. Consistent with this logic, our antibody blocking experiments implicate a role for the MCD, and not the CRD, in FHA function. However, some *B. bronchiseptica* FHA mutants, such as RBX11Δ28 and RBX11-T-E, can adhere to mammalian cells *in vitro* but display phenotypes in rats and mice that are identical to those of Δ*fhaB* strains. Thus, while lack of adherence *in vitro* may predict lack of FHA function *in vivo*, the reverse is not true; adherence *in vitro* does not necessarily reflect *in vivo* functionality. There are several possible explanations for this apparent discrepancy. It is possible that the receptors present on cells used *in vitro* represent only a subset of those that are present on relevant cells types *in vivo* and that the FHA proteins produced by RBX11Δ28 and RBX11-T-E are capable of binding to the former but not the latter. It is also possible that it is a matter of affinity; that the FHA proteins produced by RBX11Δ28 and RBX11-T-E bind receptors with an affinity that is sufficient to mediate adherence *in vitro*, but below that required for interactions that lead to tracheal colonization and immunomodulation *in vivo*. Additional scenarios can be envisaged. Regardless of the mechanistic bases, these results highlight the fact that the complexity of the *in vivo* environment cannot be accurately modelled *in vitro* at present, illustrate the challenges associated with identifying biologically relevant host cell receptors for FHA, and underscore the importance of interpreting data obtained solely from *in vitro* experiments with caution.

Our results indicate that data obtained from *in vivo* experiments deserve cautious interpretation as well. Murine lung inflammation models have proven to be useful for studying the ability of a variety of pathogens, even those causing enteric infections, to influence and/or overcome innate immunity (Philpott *et al.*, 2000; Fullner *et al.*, 2002), and they have provided insight into the function of some *B. pertussis* virulence factors (Andreasen and Carbonetti, 2008). However, the fact that Δ*fhaB* mutants are indistinguishable from wild-type *B. pertussis* in the murine lung inflammation model and the fact that *B. pertussis* fails to induce a significant anti-*Bordetella* antibody response in mice indicate that the usefulness of mouse models for studying *Bordetella* pathogenesis using *B. pertussis* is limited.

The ultimate goals of our studies on FHA are to understand how this large complex molecule contributes to the ability of bordetellae to cause respiratory disease specifically and how inflammation is controlled generally. While identifying the MCD as an important functional domain represents a significant step towards achieving those goals, it has practical implications as well. It would likely be easier and more cost-effective to produce the MCD rather than the entire mature FHA protein for inclusion in acellular pertussis vaccines. Efforts to determine if the MCD alone can be produced in large quantities, be immunogenic, and be efficacious as a vaccine are apparently already underway (Lee *et al.*, 2002; Knight *et al.*, 2006). The ability to focus on the MCD will also facilitate studies aimed at identifying host cell receptors to which FHA binds and determining the consequences of those binding events. If, as the current data suggest, FHA interacts with multiple receptors, the optimal vaccine component may be one that binds a limited set, or possibly none, of those receptors – or binds them in a way that promotes the development of a robust adaptive immune response while minimizing the inflammatory response at the immunization site. Increased incidence of pertussis in recent years attests to the need for continued vaccine development and therefore a more complete understanding of how FHA, and its various domains, function during infection.

Experimental procedures

Bacterial strains, plasmids and growth media

All strains and relevant plasmids used in this study are listed in Table S1. *Bordetella* strains were grown in Stainer–Scholte broth (Stainer and Scholte, 1970) supplemented with 100 μg μl^{-1} (2,6-*O*-dimethyl)-β-cyclodextrin or on Bordet–Gengou agar (Becton Dickinson Microbiology systems) supplemented with defibrinated sheep blood at a concentration of 7.5% (to grow *B. bronchiseptica* strains) or 15% (to grow *B. pertussis* strains). *Escherichia coli* DH5α was used for all cloning experiments and was grown in Luria–Bertani (LB) agar or broth. *E. coli* SM10λpir was used for conjugations and was grown on LB agar. Where appropriate, antibiotics were used at the following concentrations: streptomycin, 20 μg ml^{-1}; gentamicin, 20 μg ml^{-1}; ampicillin, 100 μg ml^{-1}.

To create a *B. pertussis* strain expressing FHA from *B. bronchiseptica* (Bpe138::pSJ63), the entire *fhaB* gene and promoter from *B. bronchiseptica* was cloned into a pBR322 derivative that can be used as a suicide vector in *Bordetella*, to create pSJ61. To create a plasmid in which the *B. bronchiseptica fhaB* gene was driven by the *B. pertussis fhaB* promoter, plasmid pSJ61 was digested with HindIII and Bsu36I, removing a 1.1 kb DNA fragment corresponding to the *fhaB* promoter and first 230 nucleotides of the *fhaB* gene. This fragment was replaced with the corresponding chromosomal DNA fragment from *B. pertussis* Tohama 1, to create pSJ63. This plasmid therefore contains the *B. pertussis fhaB* promoter and the first 230 nucleotides of *B. pertussis fhaB* fused in frame to the *B. bronchiseptica fhaB* gene, which results in a change of only 2 aa within the signal sequence of the *B. bronchiseptica* FhaB protein. pSJ63 was introduced into Bpe138 (a *B. pertussis* strain containing a deletion of the *fhaB* gene) and was determined via PCR analysis to have integrated into the chromosome at the *fhaB* promoter (data not shown).

The construction of strains exhibiting unmarked chromosomal deletions, rearrangements or mutations in the *fhaB* gene was done using an allelic exchange method that employs the *Bacillus subtilis sacB* gene for counterselection (Akerley *et al.*, 1995). RB50gap contains an in-frame deletion mutation of nucleotides 1449–1788 in *fhaB*. It was constructed by allelic exchange using a plasmid containing a 1 kb fragment from $fhaB_{Bp}$ that corresponds to a nearly identical sequence in $fhaB_{Bb}$ but omits the DNA corresponding to nucleotides 1449–1788 in $fhaB_{Bb}$ (because this segment is naturally missing in $fhaB_{Bp}$). $RB50CRD_{Bp}$ contains a substitution of nucleotides 3764–4178 of $fhaB_{Bb}$ with nucleotides 3422–3836 from $fhaB_{Bp}$. It was constructed using two consecutive allelic exchanges. In the first, a plasmid was constructed that contained a segment of $fhaB_{Bb}$ in which, near the centre of this fragment, nucleotides 3764–4178 were deleted. This plasmid was used to create a strain containing a deletion of nucleotides 3764–4178 in $fhaB_{Bb}$. A second allelic exchange plasmid was constructed containing a fragment of $fhaB_{Bp}$ corresponding to nucleotides 3422–3836 (these nucleotides represent the homologous sequence from $fhaB_{Bp}$ that had been deleted in $fhaB_{Bb}$ in the first allelic exchange event). Allelic exchange with this plasmid replaced the deleted nucleotides with the homologous sequence from $fhaB_{Bp}$. In a similar manner, $RB50NMCD_{Bp}$ was constructed. It contains a substitution of nucleotides 5305–6366 of $fhaB_{Bb}$ with nucleotides 4961–6036 from $fhaB_{Bp}$. In the first allelic exchange, nucleotides 5305–6366 were deleted and in the second, nucleotides 4961–6036 from $fhaB_{Bp}$ replaced the deleted nucleotides. To create RBFS10, a plasmid was constructed that contains a 1 kb DNA fragment of wild-type sequence from $fhaB_{Bp}$ centred around the site that contains the thymidine insertion mutation in RBFS4. Using allelic exchange with this plasmid, the thymidine insertion mutation was replaced by wild-type sequence, forming RBFS10. RB50RAE was constructed by allelic exchange such that the codons corresponding to aa 1213 and 1214 were changed to encode alanine and glutamic acid respectively. To create RBX11Δ28, a plasmid was constructed containing a 1 kb fragment

of DNA in which nucleotides 7287–7368 of *fhaB*$_{Bb}$, located in the centre of the fragment, were deleted in frame. Using allelic exchange with this plasmid, nucleotides 7287–7368 were deleted in *fhaB*$_{Bb}$, forming RBX11Δ28. Strain RBX11-T-E was constructed as described previously (Mazar and Cotter, 2006). All strains were confirmed to be constructed as intended by PCR and DNA sequence analysis.

Immunoblotting

Immunoblots were performed as described previously (Julio and Cotter, 2005). To evaluate expression of FHA, proteins were prepared from approximately 8.0×10^8 cfu for WCLs and 1.5×10^{10} cfu for supernatant fractions as described (Martinez de Tejada *et al.*, 1998; Julio and Cotter, 2005) and separated using 3–8% linear gradient sodium-dodecyl sulphate polyacrylamide gels, transferred to nitrocellulose (Schleicher and Schuell Bioscience), and probed with a chicken polyclonal antibody that was generated against a polypeptide corresponding to the CRD of *B. bronchiseptica* FHA (Julio and Cotter, 2005). Goat anti-chicken secondary antibody conjugated to IRdye 800 (Molecular Probes) was used to detect antigen–antibody complexes. For immunoblots evaluating the humoral response in rats and mice, whole cell proteins were run on 4–12% linear gradient SDS polyacrylamide gels, transferred to nitrocellulose, and probed with sera that had been collected 21 days post inoculation from PBS-exposed or *Bordetella*-infected rats or mice. The rat and mouse sera were diluted 1:2500. Goat anti-rat or goat anti-mouse secondary antibodies conjugated to IRdye 700 (Molecular Probes) were used to detect antigen–antibody complexes and were used at a dilution of 1:5000. Blots were visualized using an Odyssey infrared imaging system (LiCor Biosciences).

Adherence assays

Adherence of *Bordetella* to immortalized rat lung epithelial (L2) cells was performed as described (Cotter *et al.*, 1998). Bacteria were added to L2 monolayers at a moi of 200, and adherence was quantified by averaging the total number of bacterial cells and eukaryotic nuclei in two separate microscopic fields from two independent experiments. Adherence of *Bordetella* to J774A.1 macrophage-like cells was performed as described (Cotter *et al.*, 1998; Inatsuka *et al.*, 2005). For experiments using anti-CRD or anti-MCD antibodies, antibodies (diluted 1:100) were incubated with stationary phase bacteria previous to performing the adherence assay. In all adherence assays, the bacteria were observed to adhere as single cells attaching to epithelial cells or macrophages, although sometimes bacteria would be adjacent to each other. Agglutination of bacteria due to the anti-CRD or anti-MCD antibodies was not observed.

Immunostaining of whole cells

Bacterial cells from stationary phase cultures were incubated with anti-CRD (Julio and Cotter, 2005) or anti-MCD antibodies. The anti-MCD antibodies were generated in rabbits using a polypeptide corresponding to the entire MCD of FHA$_{Bb}$, which was produced in and purified from *E. coli*. Bacterial cell-antibody complexes were spotted onto a nitro-cellulose membrane (Schleicher and Schuell BioScience). The membranes were probed with either goat anti-chicken secondary antibody conjugated to IRdye 680 (for experiments using anti-CRD antibody) or goat anti-rabbit antibody conjugated to IRdye 800 (for experiments using anti-MCD antibody).

Measurement of ICAM-1 levels

Human bronchial epithelial (BEAS-2B) cells were incubated with *Bordetella* for 2 h at a moi of 100. Following several washes using PBS, the adherent bacteria were killed by gentamicin treatment for an additional 22 h. BEAS-2B cells were resuspended in PBS/3%

FBS and incubated with mouse anti-human ICAM-1 conjugated to phycoerythrin (BD Pharmigen) for 30 min at 4°C. The cells were washed twice in PBS/3% PBS, and resuspended in PBS/0.5% formaldehyde and incubated overnight at 4°C. Before analysis, cells were resuspended in PBS, filtered through a 35 μm nylon mesh, and analysed on a FACS-Aria flow cytometer (Becton Dickinson).

Animal experiments

To evaluate bacterial colonization of the respiratory tract, 3- to 4-week old female Wistar rats (Charles River Laboratories) were inoculated with 1000 cfu of *B. bronchiseptica* or 10^6 cfu of *B. pertussis* in a volume of 10 μl to the external nares. Animals were sacrificed 14 or 28 days post infection, and the number of cfu in the nasal septa and trachea was enumerated as described (Mattoo *et al.*, 2000). To evaluate the ability of bacteria to resist inflammation-mediated clearance, 3- to 4-week-old BALB/c mice (Charles River Laboratories) were inoculated intranasally with 5×10^5 cfu of *B. bronchiseptica* or *B. pertussis* in a 50 μl volume. The lungs were harvested at either 1 h, 3 days or 11 days post inoculation, and the number of cfu was enumerated from the right lung lobes as described (Mattoo *et al.*, 2000). The left lung lobes were inflated with formalin, embedded in paraffin, sectioned, stained with H&E, and examined by microscopy. SCID-Beige mice (3–4 weeks old; Charles River Laboratories) were inoculated with either 5×10^5 or 1000 cfu of *B. bronchiseptica* delivered in 50 μl or 10 μl respectively, and monitored for signs of morbidity. Mouse lung inflammation experiments have been performed multiple times for Bp536, RB50 and RBX20 and at least twice for the other strains shown in Fig. 2 with similar results. The data from one experiment are shown. We performed various animal experiments with RB50RAE once (always including RB50 and RBX9, which we have tested many times in each model) and data from a subset of those experiments are shown in Fig. 4. In every case, the results obtained with RB50RAE were indistinguishable from those obtained for RB50. In the interest of minimizing the number of animals used, we did not repeat each type of experiment with the RB50RAE mutant because we felt that, collectively, this strain had been compared with wild-type *B. bronchiseptica* multiple times and in no case was a difference apparent. RBX11Δ28 has been compared with RB50 and RBX9 in the rat and mouse models at least twice with similar results. The data from one experiment each are shown in Fig. 7. In the interest of minimizing the number of animals used, experiments with RBX9 were not carried out to day 28.

Statistical analyses

The unpaired Student's *t*-test was used for all statistical analyses except comparison of the survival data (Fig. 4C and D), for which the Log Rank (Mantel-Cox) test was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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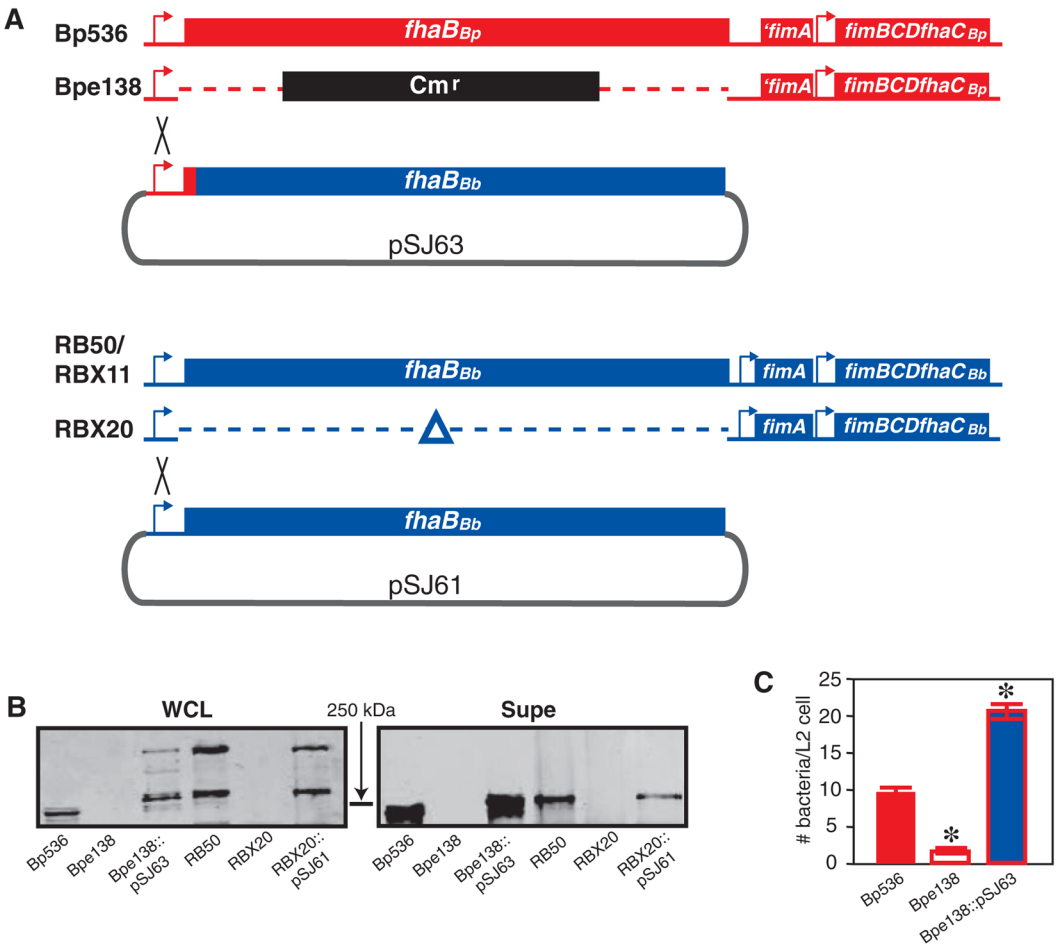

Fig. 1. Expression of *fhaB*$_{Bb}$ in *B. pertussis*

A. Schematic of strains used. The *B. pertussis fhaB* locus is shown in red and the *B. bronchiseptica fhaB* locus is shown in blue. The regions of integration of pSJ63 and pSJ61 into the chromosomes of Bpe138 and RBX20 to form Bpe138::pSJ63 and RBX20::pSJ61, respectively, are indicated by the black crosses.

B. Western blot showing FhaB (~370 kDa) and FHA (~250 kDa) in whole cell lysates (WCLs) and concentrated supernatants (Supe) of the various *B. pertussis* and *B. bronchiseptica* strains as indicated below each lane. Blots were probed with the anti-CRD antibody. The position of the 250 kDa molecular mass marker is shown.

C. Adherence of wild-type and mutant *B. pertussis* strains to L2 cells (moi = 200). Asterisks indicate a statistically significant ($P < 0.05$).

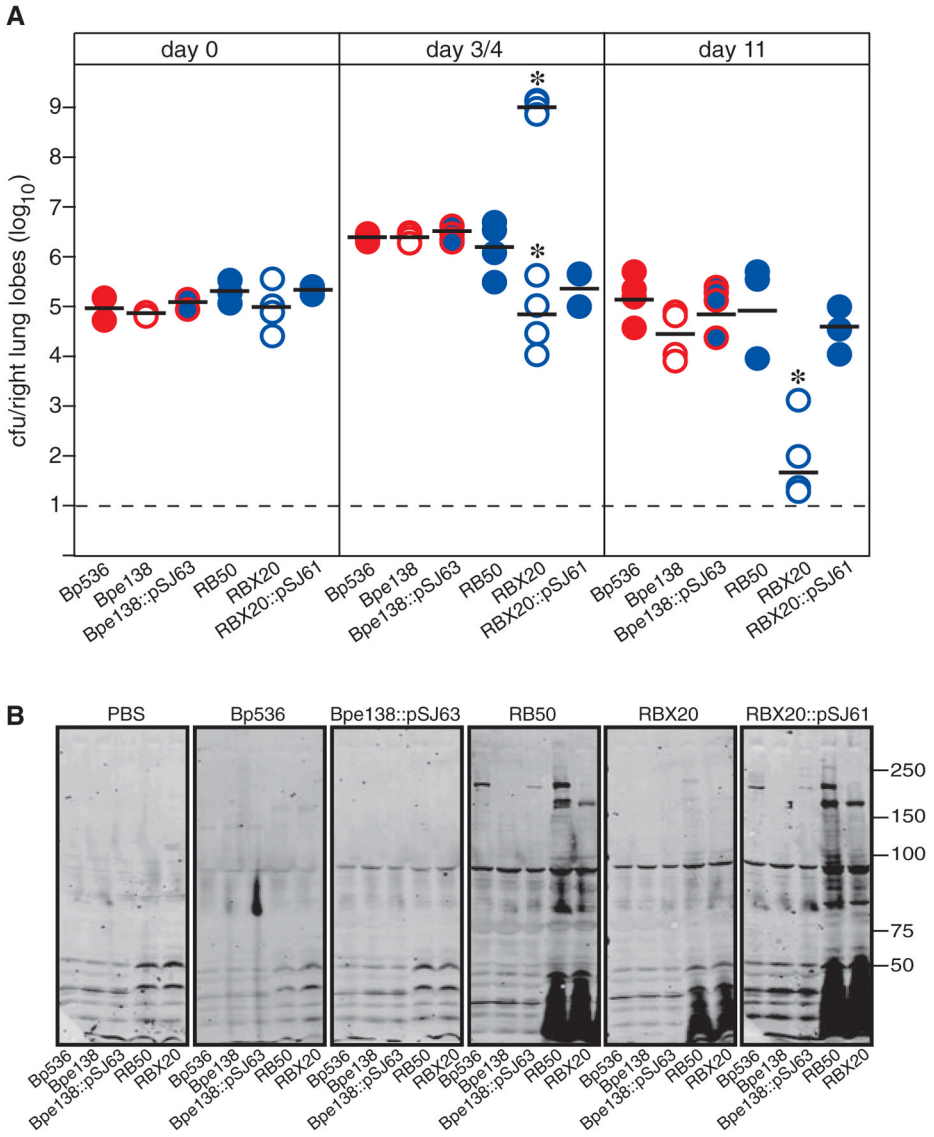

Fig. 2. Expression of *fhaB*$_{Bb}$ in *B. pertussis* does not alter its ability to infect mice

A. The number of cfu recovered from the lungs of BALB/c mice at days 0, 3 or 4, and 11 post inoculation with 50 μl PBS containing 5×10^5 cfu of the indicated strains is shown. Each circle represents the number of cfu recovered from a single animal. The horizontal black bar is the geometric mean for each group. For RBX20 at day 3/4, the means of numbers from moribund animals (~10^9) and healthy animals (~10^5) were calculated separately as shown. The dashed line represents the lower limit of detection. Asterisks indicate a statistically significant difference compared with RB50 ($P < 0.05$). This experiment has been performed at least twice with similar results. The data from one experiment are shown.

B. Western blots of whole cell lysates of the strains indicated across the bottom probed with serum from mice inoculated with PBS or the strains indicated across the top. The sera were collected 21 days post inoculation. Molecular mass markers are shown on the right.

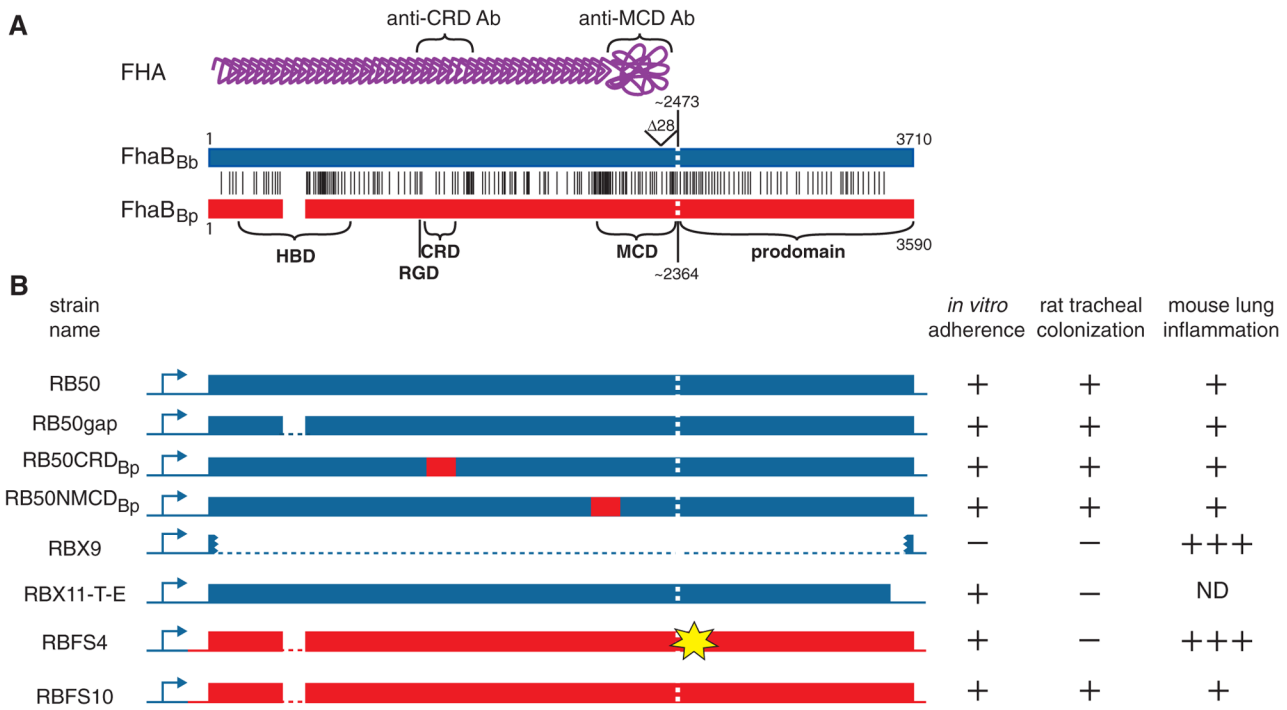

Fig. 3. A. Comparison of the FhaB proteins predicted for *B. bronchiseptica* RB50 (FhaB$_{Bb}$, blue) and *B. pertussis* Tohama 1 (FhaB$_{Bp}$, red). Vertical black lines represent the positions of amino acid differences. FhaB$_{Bb}$ contains six additional 19 aa repeats near the N-terminus (white space in FhaB$_{Bp}$). The vertical white dashed line represents the site of SphB1-dependent maturation. The various domains are designated across the bottom. A schematic of mature FHA is shown at the top in purple. The regions used for the production of the anti-CRD and anti-MCD antibodies are indicated

B. Schematic of the various 'chimeric' strains constructed and their phenotypes in adherence to L2 cells, tracheal colonization in Wistar rats, and lung inflammation in the lungs of BALB/c mice. Dark blue represents *B. bronchiseptica* RB50 DNA, red represents *B. pertussis* Tohama 1 DNA. The yellow 'star' in RBFS4 represents the location of the insertion mutation. ND, not determined. The tabular part of part B summarizes many animal experiments. Those involving strains RB50, RBX9, RBX11-T-E, RBFS4 and RBFS10 have been performed many times. Rat and mouse experiments using RB50gap, RB50CRD$_{Bp}$ and RB50NMCD$_{Bp}$ were performed once.

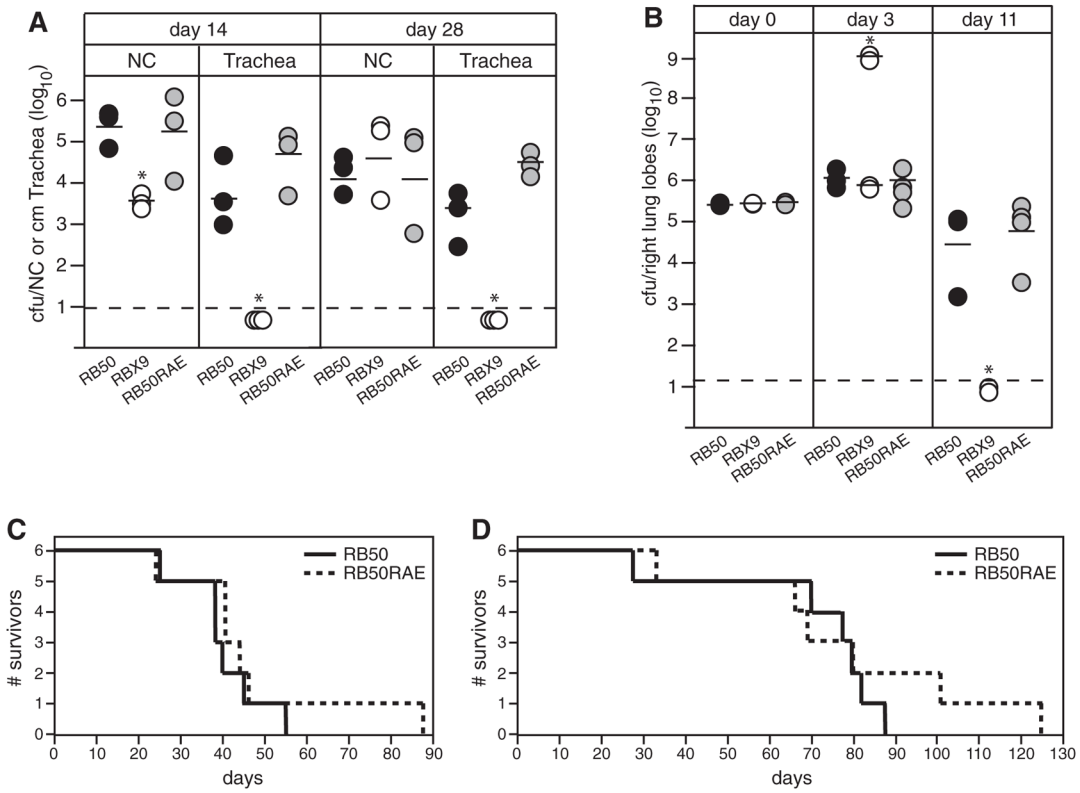

Fig. 4. Assessment of the FHA RGD triplet in *B. bronchiseptica* pathogenesis

A. Wistar rats were inoculated intranasally with 10 μl PBS containing 1000 cfu of *B. bronchiseptica* (strains indicated across the bottom) and the number of cfu recovered from the nasal cavity (NC) and 1 cm trachea determined at days 14 and 28 post inoculation. Each circle represents the number of cfu recovered from a single animal. The horizontal line shows the geometric mean for each group. The dashed line represents the lower limit of detection. *y*-axis is log scale. Asterisks indicate a statistically significant difference compared with RB50 ($P < 0.05$).

B. BALB/c mice were inoculated intranasally with 50 μl PBS containing 5×10^5 cfu of *B. bronchiseptica* (strains indicated across the bottom) and the number of cfu in the right lung lobes was determined 60 min (day 0), 3 and 11 days post inoculation. Each circle represents the number of cfu recovered from a single animal. The horizontal line shows the geometric mean for each group. The dashed line represents the lower limit of detection. For RBX9, the means at day 3 for moribund (~10^9) and healthy (~10^6) animals were calculated separately as indicated. *y*-axis is log scale. Asterisks indicate a statistically significant difference compared with RB50 ($P < 0.05$).

C and D. SCID/Bg mice were inoculated intranasally with 50 μl PBS containing 5×10^5 (for C) or 10 μl containing 1000 (for D) cfu of RB50 (solid line) or RB50RAE (dashed line) and the number of survivors plotted over time. The mean time to death was not significantly different between RB50 and RB50RAE for either experiment, as determined by the Log Rank (Mantel-Cox) test. RB50 and RBX9 have been compared in these models many times with similar results. Each type of animal experiment that included the RB50RAE strain was performed once and not repeated in the interest of minimizing the number of animals used because no difference between RB50RAE and RB50 was detected in any of the experiments.

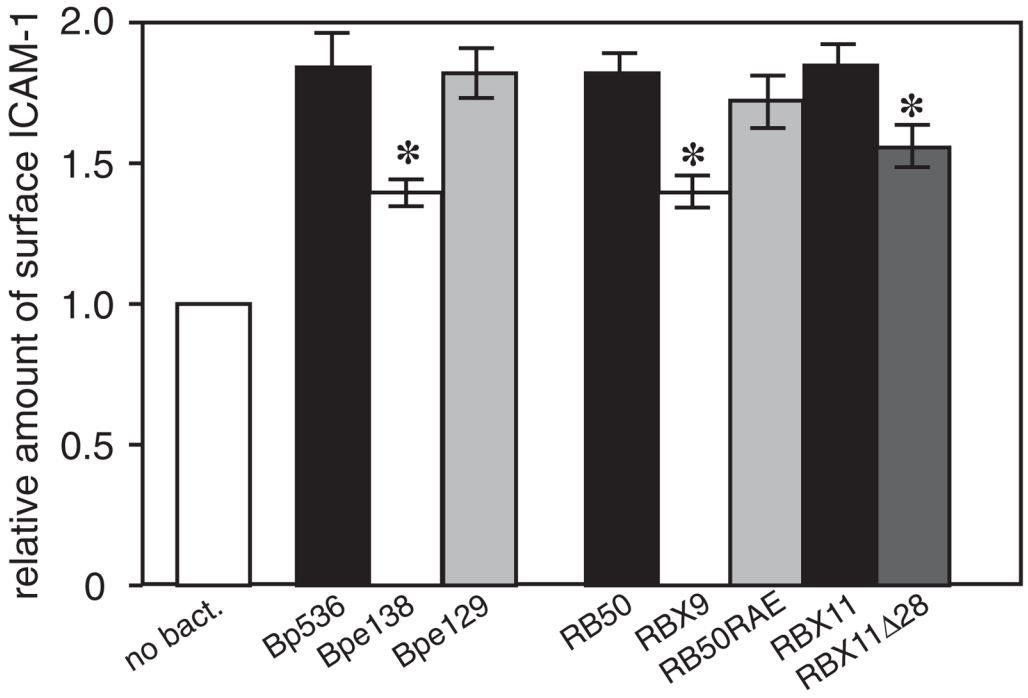

Fig. 5.

ICAM-1 induction. BEAS-2B cells were incubated with the indicated strains of *B. bronchiseptica* or *B. pertussis* at a moi of 100 and the amount of surface ICAM-1 was determined by flow cytometry. (Bp536 = wild-type *B. pertussis*, Bpe138 = *B. pertussis* Δ*fhaB*, Bpe129 = *B. pertussis* RAD mutant, RB50 = wild-type *B. bronchiseptica*, RBX9 = RB50Δ*fhaB*, RB50RAE = RB50 RAE mutant, RBX11 = RB50Δ*fhaS*, RBX11Δ28 = RB50 containing the FHA 'Δ28' mutation.) Fluorescence of cells incubated with PBS alone was set as a value of one and relative levels of fluorescence in samples incubated with bacteria are shown. Asterisks indicate a statistically significant difference compared with the wild-type parental strain ($P < 0.05$).

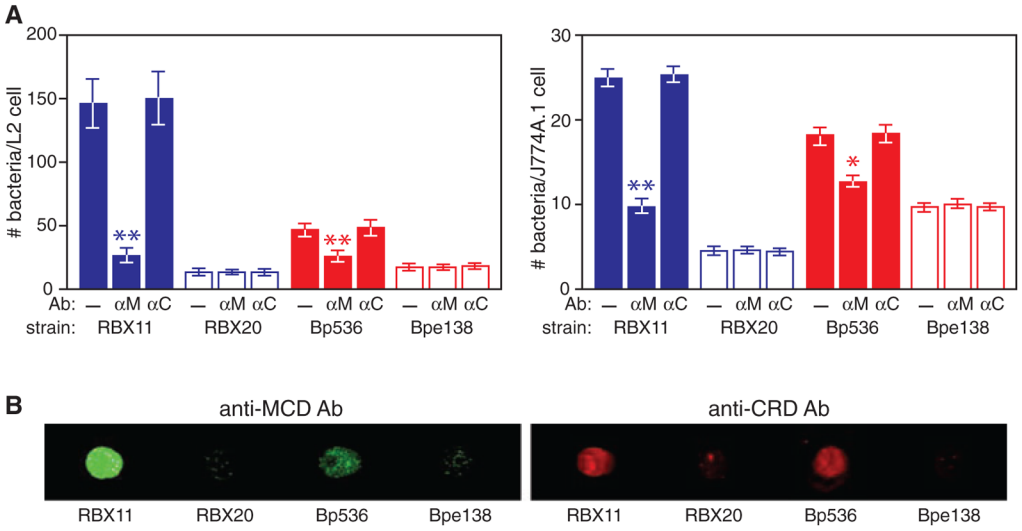

Fig. 6. Antibodies to the MCD, but not the CRD, block adherence to L2 and J774A.1 cells

A. *B. pertussis* and *B. bronchiseptica* strains (indicated) were incubated with anti-MCD (αM) or anti-CRD (αC) antibodies then tested for adherence to L2 or J774A.1 cells. The number of bacteria per cell is shown. Asterisks indicate statistically significant differences in adherence compared with wild-type bacteria that were not incubated with antibody. $*P < 0.05$ and $**P < 0.01$.

B. Aliquots of the bacteria used in the adherence assays were incubated with goat anti-chicken antibody conjugated to IRdye 680 (for anti-CRD) or goat anti-rabbit conjugated to IRdye 800 (for anti-MCD), washed, then spotted into nitrocellulose and fluorescence was visualized using an Odyssey imager.

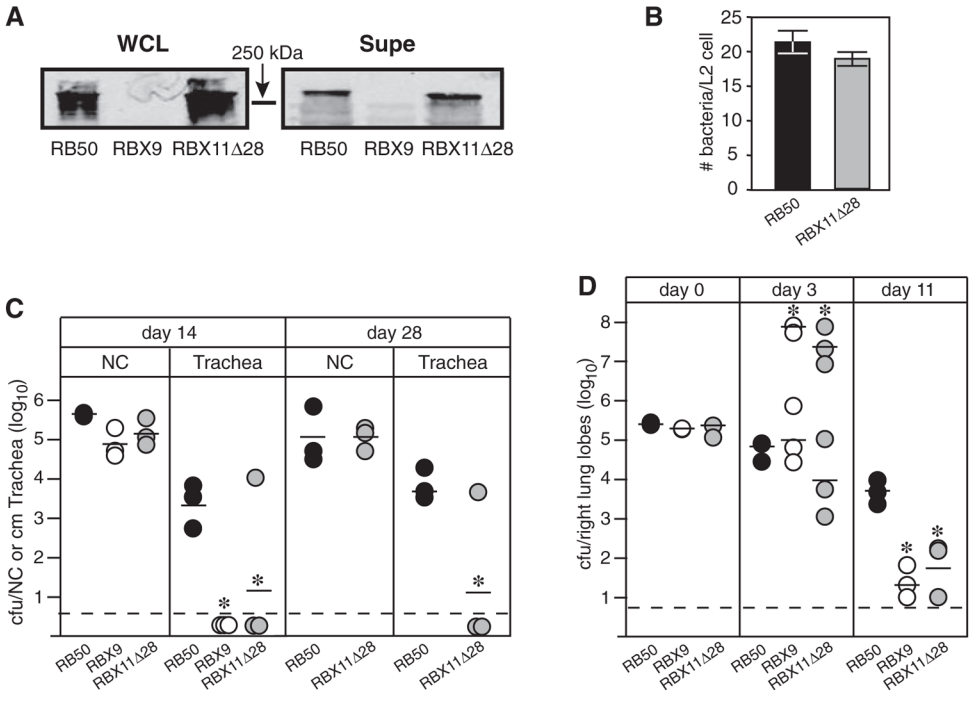

Fig. 7. The MCD is required for FHA function *in vivo*

A. Western blot showing FHA (~250 kDa) in whole cell lysates (WCLs) and concentrated supernatants (Supe) of the indicated *B. bronchiseptica* strains. Blots were probed with the anti-CRD antibody.

B. Adherence of RB50 and RBX11Δ28 to L2 cells is shown. Moi = 200. The difference between the values was not statistically significant.

C. Wistar rats were inoculated intranasally with 10 μl PBS containing 1000 cfu of *B. bronchiseptica* (strains indicated across the bottom) and the number of cfu recovered from the nasal cavity and 1 cm trachea determined at days 14 and 28 post inoculation. Each circle represents the number of cfu recovered from a single animal. The horizontal line shows the geometric mean for each group. The dashed line represents the lower limit of detection. *y*-axis is log scale. $*P < 0.05$.

D. BALB/c mice were inoculated intranasally with 50 μl PBS containing 5×10^5 cfu of *B. bronchiseptica* (strains indicated across the bottom) and the number of cfu in the right lung lobes was determined 60 min (day 0), 3 and 11 days post inoculation. Each circle represents the number of cfu recovered from a single animal. The horizontal line shows the geometric mean for each group. The dashed line represents the lower limit of detection. For RBX9 and RBX11Δ28, the means at day 3 for moribund (~10^8) and healthy (~$10^{4–5}$) animals were calculated separately as indicated. *y*-axis is log scale. $*P < 0.05$. The rat and mouse experiments using RBX11Δ28 were performed at least twice with similar results and the data from one of each type of experiment are shown.