

Molecular characterization of *Bordetella bronchiseptica* filamentous haemagglutinin and its secretion machinery

Françoise Jacob-Dubuisson,¹ Bettina Kehoe,^{3,4} Eve Willery,¹
Nathalie Reveneau,^{1,2} Camille Locht¹ and David A. Relman^{3,4}

Author for correspondence: David A. Relman. Tel: +1 650 852 3308. Fax: +1 650 852 3291.
e-mail: relman@cmgm.stanford.edu

^{1,2} INSERM U447, Institut de Biologie de Lille¹ and Département de Microbiologie des Ecosystèmes, Institut Pasteur de Lille², 1 rue Calmette, F-59019 Lille Cedex, France

³ Departments of Medicine and Microbiology & Immunology, Stanford University, Stanford, CA 94305, USA

⁴ VA Palo Alto Health Care System 154T, 3801 Miranda Avenue, Palo Alto, CA 94304, USA

Two closely related pathogens, *Bordetella pertussis* and *Bordetella bronchiseptica*, share a number of virulence factors. Filamentous haemagglutinin (FHA) is widely regarded as the dominant adhesin of *B. pertussis*, and its multiple binding activities have been well characterized. This large protein is produced and secreted at high levels by *B. pertussis* and significantly lower levels by *B. bronchiseptica* strains. FHA secretion is mediated by a single outer-membrane accessory protein, FhaC. The genes encoding FHA and FhaC in *B. bronchiseptica* were characterized by sequencing and functional analyses and are highly similar to those of *B. pertussis*. The most distinctive feature of *B. bronchiseptica* FHA is additional repeats in the N-terminal portion of the predicted protein. Interestingly, a point mutation in the *fhaB* promoter region of the *B. bronchiseptica* GP1 isolate, relative to other isolates, was found to be detrimental to promoter activity and to FHA production. FhaC and the N-terminal secretion domain of FHA of *B. bronchiseptica* were fully functional for secretion in *B. pertussis*. Thus, the different levels of FHA secretion by these *Bordetella* species might reflect differences in physiology, composition and structure of cell envelope, or differential protein degradation. Characterization of FHA expression and function may provide clues as to the basis of host species tropism, tissue localization and receptor recognition.

Keywords: *Bordetella bronchiseptica*, filamentous haemagglutinin, FHA, secretion, adherence

INTRODUCTION

Bordetella species are small, Gram-negative coccobacilli that cause respiratory tract infections in humans, animals and birds. A strictly human pathogen, *Bordetella pertussis* causes whooping cough, a highly contagious respiratory disease with severe clinical manifestations in children (Hewlett, 1997; Rappuoli, 1994). *Bordetella parapertussis* is associated with milder forms of whooping cough in humans; *B. parapertussis* strains are also found in sheep (Porter *et al.*, 1994). *Bordetella*

bronchiseptica is uncommon in humans (Stefanelli *et al.*, 1997), but it is a frequent cause of respiratory diseases and asymptomatic infections in several other mammals (Goodnow, 1980).

These three closely related organisms produce similar arrays of virulence factors (Rappuoli, 1994). Among these, filamentous haemagglutinin (FHA) is regarded as the dominant attachment factor (Arico *et al.*, 1993; Leininger *et al.*, 1993; Relman *et al.*, 1989; Urisu *et al.*, 1986). *B. pertussis* FHA (FHA Bp) is also highly immunogenic in humans and is a protective antigen in animal models (Amsbaugh *et al.*, 1993; Cahill *et al.*, 1993; Shahin *et al.*, 1992). The multiple binding activities of the mature 220 kDa protein have been the focus of many studies. FHA is recognized by lactose-containing glycolipids on ciliated respiratory epithelial cells

Abbreviations: FHA, filamentous haemagglutinin; GFP, green fluorescent protein.

The GenBank accession numbers for the sequences reported in this paper are AF111794, AF111796, AF111797 and AF111798.

(Tuomanen *et al.*, 1988). It binds to sulphated carbohydrates of sulphatides and proteoglycans on the surface of epithelial cells or in the extracellular matrix (Brennan *et al.*, 1991; Menozzi *et al.*, 1991, 1994a). In addition, it possesses an RGD motif, which is a canonical recognition sequence for members of the integrin family. This FHA RGD site within the mature protein is recognized by the beta-3 integrin, leucocyte response integrin, in concert with integrin-associated protein (Ishibashi *et al.*, 1994; Relman *et al.*, 1990). As the dominant adhesin, differences in FHA-mediated function might contribute to differences in *Bordetella* host species tropism.

B. bronchiseptica FHA (FHA Bb) has a molecular mass and haemagglutination properties that are similar to those of FHA Bp (Sakurai *et al.*, 1993). Electron microscopy studies of FHA Bb preparations suggest similar structure and dimensions to those of FHA Bp (Ohgitali *et al.*, 1991). Most monoclonal antibodies generated against FHA Bp cross-react with FHA Bb, indicating shared epitopes (Menozzi *et al.*, 1994a, b). FHA Bb is required for *B. bronchiseptica* colonization of the rat trachea (Cotter *et al.*, 1998).

A noteworthy feature of FHA is its high level of secretion by *B. pertussis* (Locht *et al.*, 1993). FHA represents the most abundant polypeptide in the culture supernatant of *B. pertussis* grown *in vitro*. It is also associated with the bacterial outer membrane. Coating of the bacterial outer surface with FHA is thought to be responsible for autoagglutination of the bacteria by FHA–FHA homotypic interactions (Menozzi *et al.*, 1994b).

In *B. pertussis*, the mature, 220 kDa, form of FHA derives from a 370 kDa FhaB precursor by an as-yet-uncharacterized proteolytic removal of the large C-terminal portion (Domenighini *et al.*, 1990; Renaud-Mongenie *et al.*, 1996). FHA is exported by a signal-peptide-dependent pathway across the cytoplasmic membrane and it requires a single specific accessory protein, FhaC, for translocation across the outer membrane (Jacob-Dubuisson *et al.*, 1996; Lambert-Buisine *et al.*, 1998; Willems *et al.*, 1994). An N-proximal 115-residue-long region of FHA, called the secretion domain, is essential for FHA secretion (Jacob-Dubuisson *et al.*, 1997). This region probably interacts with FhaC in a specific manner to drive translocation of FHA through the outer membrane. The molecular details of this step are still under investigation. FHA appears to cross both membranes in a coupled fashion and acquires its native conformation upon extrusion from the outer membrane (Guédin *et al.*, 1998).

FHA appears to be produced and/or secreted at lower levels by *B. bronchiseptica* than by *B. pertussis* (Leininger *et al.*, 1993; Menozzi *et al.*, 1994a). Furthermore, as a dominant adherence factor, FHA may play a role in differential host species tropism and receptor recognition. In this study, we set out to characterize the genetic basis for *B. bronchiseptica* FHA expression and secretion, compare these findings with

the corresponding features in *B. pertussis*, and explore some of the possible factors responsible for differences in FHA expression by these two species.

METHODS

Bacterial strains and plasmids. *B. bronchiseptica* GP1 was originally isolated from a guinea pig (Akerley *et al.*, 1992). *B. bronchiseptica* BB1015 (Menozzi *et al.*, 1991), RB50 (Cotter & Miller, 1994), NL1013, S87, 899L and NL1011 (Antoine & Loch, 1992) and *B. pertussis* BP536 (Relman *et al.*, 1989), BPSM (Menozzi *et al.*, 1994a), BPGR4 ($\Delta fhaB$) (Locht *et al.*, 1992) and BPEC ($\Delta fhaC$) (Guédin *et al.*, 1998) have been described earlier. *B. parapertussis* PEP is a clinical isolate (Nordmann *et al.*, 1992) and *B. parapertussis* 8234 was a gift from E. Hewlett, University of Virginia.

Bordetella culture conditions were as described by Loch *et al.* (1992). Phenotypic modulation of GP1 was achieved by the addition of 20 mM MgSO₄ to modified Stainer–Scholte (SS) media, or by incubation at room temperature of Bordet–Gengou agar plates.

DNA cloning and other manipulations. A chromosomal library from *B. bronchiseptica* GP1 was created by partial *Sau3AI* restriction of genomic DNA, followed by ligation with *Bam*HI-restricted pHC79 (Hohn & Collins, 1980), and transformation of *Escherichia coli* DH5 α . Cosmid gp1#4 was selected after recognition by colony hybridization with a *bugAS* gene probe. An *Eco*RI restriction map suggested that this cosmid also included the *fhaB* gene. *Eco*RI restriction fragments of cosmid gp1#4 were subcloned into pBSKSII (Stratagene). pBK117 consists of the pBSKII+ vector plus a 10 kb *Eco*RI insert comprising the 5' end of *B. bronchiseptica fhaB* subcloned from cosmid gp1#4. pBK116 consists of the same vector with an additional, naturally occurring 1.2 kb *Eco*RI fragment from gp1#4 containing the 3' end of *B. bronchiseptica fhaB*. pBK123 contains a 5 kb *Bam*HI insert corresponding to the 3' half of the 10 kb *Eco*RI insert of pBK117. These plasmid inserts were further subcloned, and both strands were sequenced using a variety of internal primers. Independent amplification and subcloning of specific regions were performed to confirm the sequence at ambiguous positions. DNA sequence analysis of pBK123 indicated the absence of two Gs at positions 5561 and 9764 relative to the *B. pertussis fhaB* sequence; these deletions result in frameshifts in the *B. bronchiseptica fhaB* sequence, leading to premature termination of the predicted protein. Direct PCR amplification of these regions from the chromosome of GP1 and several other *B. bronchiseptica* isolates showed that both missing nucleotides were present in all the strains. Therefore, we concluded that their absence in pBK123 was due to either cloning artefacts or sequencing errors.

The *fhaB*–*gfp* fusions were generated as follows. The parent vector, pBK152, was constructed by insertion of a promoterless 729 bp *gfp*mut3 fragment from an *Eco*RI/*Hind*III digest of pGFPmut3 into pBBR1MCS-5 (Cormack *et al.*, 1996; Kovach *et al.*, 1994). Segments (320 bp) of DNA encompassing the promoter region of *fhaB* were amplified by PCR using chromosomal DNA from the indicated strains and the primers FHA-192FR (5'-GGAAAATTCTGAATTCCCGCGC-3') and FHA328RR (5'-CGGTGgAAATtCTCGCTCACGG-3'). The *Eco*RI sites (underlined) were used for the cloning of the promoter regions upstream of the green fluorescent protein (GFP) coding sequence in pBK152; the lower case letters in FHA328RR reflect nucleotide changes for incorporation of an *Eco*RI site. The PCR was performed using

30 cycles of 94 °C for 30 s, 45 °C for 30 s and 72 °C for 30 s. The reaction mixture included a final concentration of 5% (v/v) glycerol. Proper orientation of the inserts was confirmed by restriction enzyme digestion and sequence analysis. At least two independent clones were completely sequenced for each construct to detect and avoid errors due to the PCR. Plasmids were transformed into *Escherichia coli* SM10 and introduced by conjugation into *B. bronchiseptica* GP1 and *B. pertussis* BPGR4. These strains were grown in modified SS media supplemented with 100 µg streptomycin ml⁻¹ and 20 µg gentamicin ml⁻¹ at room temperature, at 37 °C or at 37 °C in the presence of 20 mM MgSO₄.

pBG4 was described earlier (Renauld-Mongenie *et al.*, 1996). It contains a 2.8 kb *EcoRI*–*Bam*HI fragment comprising the first third of *B. pertussis* *fhaB* encoding Fha44. pEC40 encodes the *B. bronchiseptica* Fha44. It was generated using the same procedure as described for pBG4 (Renauld-Mongenie *et al.*, 1996). Briefly, the 10 kb *EcoRI* fragment of pBK117 was cloned into pBBR122, a vector that is able to replicate in *Bordetella* spp. (Antoine & Lochter, 1992). The internal 7.1 kb *Bam*HI fragment was then deleted by digesting the resulting plasmid with *Bam*HI and re-ligating it, thereby yielding pEC40.

pFJD16Δ was obtained as follows. The unique *PvuI* site of pBBR1MCS was removed using a T4 polymerase treatment of the *PvuI*-restricted plasmid, followed by re-ligation. The 2.2 kb *Sall*–*XbaI* fragment from pFJD16 (Jacob-Dubuisson *et al.*, 1996), encoding *B. pertussis* *fhaC*, was then introduced into the corresponding sites of the modified pBBR1MCS, giving rise to pFJD16Δ. For the expression of *B. bronchiseptica* *fhaC*, the 1.7 kb *PvuI*–*SacI* *fhaC* fragment of pFJD16Δ was replaced by the corresponding fragment of *B. bronchiseptica* *fhaC*, giving rise to pEC46 (*SacI* site was located within vector polylinker). This construct was verified by the absence of two restriction sites in *B. bronchiseptica* *fhaC* that are present in *B. pertussis* *fhaC*, and was partly sequenced to confirm the gene replacement. It should be noted that although this cloning procedure resulted in the replacement of the first 44 residues of FhaC Bb by those of FhaC Bp, sequence data indicate that only one substitution occurs in that region (Willems *et al.*, 1993 and results presented in this paper).

The *B. bronchiseptica* FhaC coding sequence was PCR amplified from *B. bronchiseptica* GP1 chromosomal DNA prepared with the Qiagen Genomic DNA kit, using the oligonucleotides 5'-ATGACTGACGCAACGAACCGTTTCC-3' and 5'-GCGTTCTCGCCGGGCTCAGAACTG-3' as primers. The amplicon was cloned into *EcoRV*-restricted pZERO (Invitrogen) and sequenced on both strands using the universal and reverse vector-based primers, as well as several internal primers. Two independent clones were sequenced entirely and a third clone was sequenced partially as the sequences of the first two differed at two positions.

Sequencing strategy. pBK117, pBK123 and pBK116 were used as templates for the sequencing of *B. bronchiseptica* *fhaB*. The *fhaB* gene was sequenced by using the universal and reverse M13 vector-based primers, as well as a series of internal primers that were designed based on the sequences generated. Sequencing was performed using ABI Prism 377 and ABI 373 DNA Sequencers and the kits supplied by the manufacturer (Perkin Elmer). Alignments of sequences and generation of contigs were performed using the DNASTAR program and ABI Sequence Navigator and AutoAssembler (Perkin Elmer). The database accession numbers are as follows: *B. bronchiseptica* GP1 *fhaB*, AF111796; *B. bronchiseptica* GP1 *fhaC*, AF111794;

B. bronchiseptica RB50 upstream region of *fhaB*, AF111797; *B. parapertussis* 8234 region upstream of *fhaB*, AF111798.

Measurement of GFP activity. GP1 or BPGR4 cells containing *fha*–*gfp* fusion plasmids were swabbed from plates into modified SS media or taken directly from liquid cultures and diluted to an OD₆₀₀ of 0.05. Cells were analysed during log and stationary phases and under modulating (20 mM MgSO₄ or room temperature) and non-modulating conditions. Cells were fixed in 1% paraformaldehyde in PBS. Median fluorescence at 488 nm was measured from 10000 cells that were gated from 10 to 10000 units (Becton Dickinson FACScan). Four independent exconjugants for each plasmid construct were analysed separately on each of three occasions. The mean value of median fluorescence from each of these occasions was calculated.

Protein analyses. Proteins from supernatants or cell extracts were analysed by SDS-PAGE on 8 or 10% polyacrylamide gels and stained with Coomassie brilliant blue, or transferred to membranes and probed with antibody. For the comparison of FHA production/secretion between the different strains, haemagglutination assays on fresh culture supernatants or intact cells were performed as described previously (Jacob-Dubuisson *et al.*, 1996). ELISA was performed as described previously (Jacob-Dubuisson *et al.*, 1996). The titres of the supernatants corresponded to the dilution yielding an A₄₂₀ value threefold higher than the background value. Polyclonal chicken anti-FHA IgY and polyclonal anti-FhaC rat IgG were made by Eurogentec. FHA and Fha44 were purified from BPGR4 culture supernatants by heparin-Sepharose chromatography as described by Menozzi *et al.* (1991). Membrane extracts for the detection of FhaC were prepared as described by Jacob-Dubuisson *et al.* (1996) except that the Sarkosyl extraction step was omitted. N-terminal sequencing was performed at the CNRS URA1309, Institut Pasteur de Lille. Sample preparation was as described previously (Jacob-Dubuisson *et al.*, 1996).

RESULTS

Primary structure of the *B. bronchiseptica* GP1 FHA structural gene

B. bronchiseptica GP1, a naturally occurring isolate from a laboratory-reared guinea pig (Akerley *et al.*, 1992), produces and secretes FHA at much lower levels than *B. pertussis* BPSM or *B. pertussis* 18323 (Fig. 1). Other *B. bronchiseptica* strains, such as BB1015 (Fig. 1) or RB50 (not shown), produce intermediate levels of FHA. Three procedures were used to assess the levels of FHA secretion by *B. bronchiseptica* BB1015, RB50 and GP1 as compared to that of *B. pertussis* BPSM. First, unconcentrated culture supernatants of the three *B. bronchiseptica* isolates grown to equivalent cell densities were analysed by SDS-PAGE and Coomassie blue staining of the gels. Serial dilutions of the BPSM supernatants were loaded for comparison. Based on densitometric scanning, BB1015 and RB50 secrete approximately eightfold lower amounts of FHA than does BPSM. The amount of FHA secreted by GP1 is even lower, and is barely detectable on the gel (Fig. 1). Quantification of the levels of secreted FHA using both ELISA and haemagglutination assays indicated that the titres of FHA in RB50 and BB1015 supernatants are

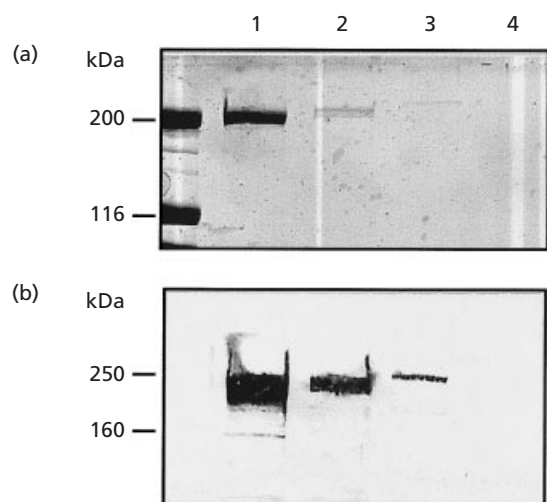


Fig. 1. FHA secretion by *B. pertussis* and *B. bronchiseptica*. Unconcentrated culture supernatants (late exponential phase) of *B. pertussis* BPSM (lanes 1), *B. pertussis* 18323 (lanes 2), *B. bronchiseptica* BB1015 (lanes 3) and *B. bronchiseptica* GP1 (lanes 4) were analysed by SDS-PAGE and stained with Coomassie brilliant blue (a), or transferred to a nitrocellulose membrane and probed by immunoblotting with anti-FHA IgY (b). Note that FHA Bb migrates slightly slower than FHA Bp. Molecular masses of size markers are indicated on the left.

reproducibly four- to eightfold lower than those of BPSM, whereas the amount of FHA in GP1 supernatants is too low for detection by these techniques. Nevertheless, *B. bronchiseptica* GP1 does secrete FHA, as some FHA was obtained from GP1 culture supernatants following concentration on heparin-Sepharose (not shown). Interestingly, the amounts of cell-associated FHA in all four isolates roughly paralleled those of FHA released in the culture supernatants, as assessed by immunoblot analysis of cell extracts and by the haemagglutination titres of intact cells (not shown). Therefore, *B. bronchiseptica* BB1015 and RB50 can be considered intermediate FHA producers/secretors and GP1 a low producer/secretor, as compared to *B. pertussis*.

To determine whether these differences in production and secretion might be explained by the sequence of the FHA structural gene, the *fhaB* gene was cloned from *B. bronchiseptica* GP1 and sequenced entirely. An 11370 bp DNA primary structure was determined, encompassing the *fhaB* promoter region, the FHA-encoding segment, and a short 3' non-coding region. The GP1 *fhaB* ORF is 10902 bp in length, in contrast to the 10770 bp *fhaB* gene from *B. pertussis* BP536 (corrected, see below) (Domenighini *et al.*, 1990; Relman *et al.*, 1989). The G + C content of this ORF is 67.7 mol%. Sequence alignments of the *B. pertussis* and *B. bronchiseptica* *fhaB* genes revealed that the DNA sequences are 92.8% identical. The most important difference is an insertion of 114 nt in *B. bronchiseptica* *fhaB* at nucleotide position 1404 (the numbers are given relative to the initiation codon). There are also several

additional insertions and deletions of substantially smaller sizes (see Fig. 2).

At the 3' end of the GP1 *fhaB* ORF, after nucleotide 10849, an extra G was detected, relative to the previously published *B. pertussis* sequence. This insertion is predicted to result in a frameshift and termination of the ORF 4 nt prior to the end of the published *fhaB* sequence. To investigate this discrepancy further, the corresponding *fhaB* DNA segments were amplified directly from the chromosome of GP1 and from *B. bronchiseptica* isolates BB1015, NL1013, S87 and 899L. The sequence of these amplified regions confirmed the presence of an extra G at this position. This result prompted us to re-examine the *B. pertussis* *fhaB* sequence. PCR amplification and sequencing of this same region from the chromosome of *B. pertussis* BPSM clearly showed the presence of a G that is missing in the published *B. pertussis* *fhaB* sequence. Consequently, the previously published, predicted C-terminal sequence of FHA Bp (RLRSRISAARTTGSSMKPTNR) should be replaced beginning at residue 3571 with RLRVED-IGGKNYRVFYETNK, resulting in an FHA polypeptide one residue shorter (3590 amino acids) than that originally predicted (Domenighini *et al.*, 1990). The *B. pertussis* *fhaB* database file, X52156, has been corrected accordingly.

The DNA sequences downstream of the *B. pertussis* and *B. bronchiseptica* *fhaB* genes diverge significantly 69 nt after the stop codon (not shown). The conserved 3' untranslated segment includes a 9 bp perfect inverted repeat both in *B. bronchiseptica* and in *B. pertussis*, potentially acting as a rho-independent terminator for *fhaB* transcription (Domenighini *et al.*, 1990; Willems *et al.*, 1992). All five *B. bronchiseptica* isolates sequenced in that region are identical, except for two positions. In addition, we sequenced a 44 bp region encompassing the 3' end of the *fhaB* gene and the region immediately downstream of the termination codon from the *B. paraptentis* PEP clinical isolate. This sequence was also identical to the corresponding region of the *B. bronchiseptica* GP1 genome (not shown). The sequence divergence observed 69 nt after the *fhaB* stop codon between *B. bronchiseptica* and *B. paraptentis* on the one hand, and *B. pertussis* on the other, is consistent with the recently reported 419 bp insertion corresponding to the beginning of a complete *fimA* gene present in *B. bronchiseptica* and *B. paraptentis*, which is truncated in *B. pertussis* (Boschwitz *et al.*, 1997).

Analysis of the predicted FHA protein sequence

FHA Bb is predicted to comprise 3634 residues, with an amino acid composition and general sequence features very similar to those of FHA Bp (Domenighini *et al.*, 1990). The two proteins are 93.5% identical, with a total of 44 additional residues in the FHA Bb protein (Fig. 2). This high level of amino acid identity occurs throughout the *B. bronchiseptica* FHA sequence and is not restricted to the portion encoding the mature protein (approx. 2199 amino acids beginning at the N terminus).

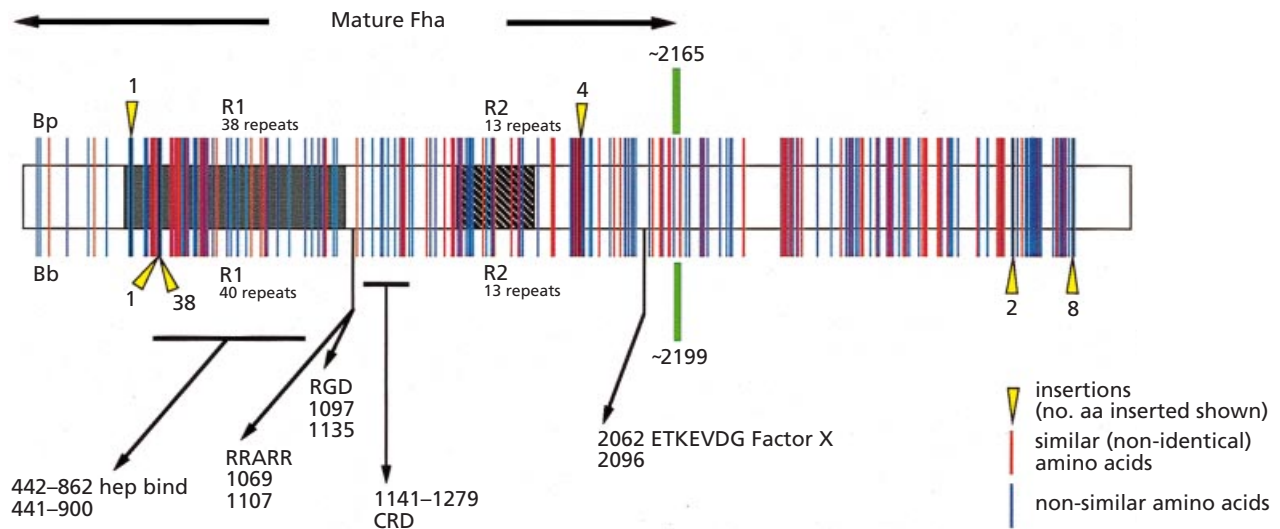


Fig. 2. Schematic representation of the alignment of the predicted *B. pertussis* BP536 and *B. bronchiseptica* GP1 FHA sequences. The long, thick rectangle represents the entire precursor protein; numbers at the top correspond to the *B. pertussis* BP536 published (corrected) sequence (Bp), and numbers on the bottom to the *B. bronchiseptica* GP1 sequence (Bb). The cleavage site that creates the mature FHA protein is marked by the thick green line. The plain and hatched grey rectangles represent the R1 and R2 repeat regions, respectively, with the number of 19-residue repeats indicated for each protein. The positions of domains involved in FHA adhesion, including a heparin-binding domain (hep bind), an RGD sequence, a carbohydrate-recognition domain (CRD) and a sequence homologous to Factor X are indicated with Bp (top) and Bb (below) FHA amino acid positions.

The most striking difference between the two proteins lies in the R1 repeat region, between residues 343 and 900 (Fig. 2), which has been proposed to form one side of the β hairpin structure of FHA (Makhov *et al.*, 1994). In the R1 region, FHA Bb contains 40 tandem copies of a 19-residue repeat, two more than in FHA Bp (Fig. 2). The additional two copies are inserted between the seventh and the eighth repeats as defined by Makhov *et al.* (1994). Both proteins contain 13 tandem copies of a 19-residue repeat in the R2 region, which is located between residues 1475 and 1727 in FHA Bb (Fig. 2). As in FHA Bp, sequences of the *B. bronchiseptica* repeats all fit the R1 and R2 consensus sequences defined by Makhov *et al.* (1994), although no residue is strictly conserved within each group.

All the features of FHA Bp with demonstrated or putative biological function are conserved in the predicted FHA Bb. Both RGD sequences, one in the mature FHA sequence (residues 1097–1099) and the other in the C-terminal processed region of the precursor, are predicted to be present at identical positions in the FHA Bb sequence. Other FHA domains with putative adhesin activity, the heparin-binding domain (FHA Bp residues 442–862) (Hannah *et al.*, 1994) and the putative carbohydrate-binding domain (positions 1141–1279) (Liu *et al.*, 1997; Prasad *et al.*, 1993) are more than 90 % identical between the two proteins. The ETKEVDG sequence (positions 2096–2102) with a possible role in integrin recognition of FHA (Rozdzinski *et al.*, 1995), is identical between the two proteins. An additional feature common to both proteins is an arginine-rich region (RRARR), which serves as a proteolytic cleavage

site in mature FHA (Delisse-Gathoye *et al.*, 1990; Domenighini *et al.*, 1990; Relman *et al.*, 1989). The region between FHA Bp residues 1929 and 2019 and, in particular, residues 2001–2015, contains linear epitopes that are dominant in eliciting an antibody response to FHA (Leininger *et al.*, 1993; Wilson *et al.*, 1998); 7 of 91 and 2 of 15 residues differ within these segments, respectively, between the two FHA homologues.

The sequence regions that are important for the secretion of FHA are strictly conserved between the two protein homologues. The NPNL and NPNG motifs shown to be critical for FHA Bp secretion (Jacob-Dubuisson *et al.*, 1997) are also present in FHA Bb. The 71-residue signal peptides are identical with the exception of two residues. The N-terminal residue of mature FHA Bp has recently been determined to be a modified glutamine, encoded by the 72nd codon after the initiation codon (Lambert-Buisine *et al.*, 1998). To determine the first residue of FHA Bb, we overproduced a truncated, N-terminal portion of the GP1 FHA in *B. pertussis*. This protein, which is the *B. bronchiseptica* equivalent of Fha44 (Renauld-Mongenien *et al.*, 1996), was efficiently secreted in *B. pertussis*, suggesting that the secretion signals of FHA Bb are fully functional in the heterologous host. In a fashion similar to that of *B. pertussis* Fha44, most of *B. bronchiseptica* Fha44 was not amenable to N-terminal Edman degradation, suggesting that the N-terminal residue of the *B. bronchiseptica* FHA is also blocked. In the first few cycles, minute chromatographic peaks corresponding to the QGLVP sequence were detectable (data not shown),

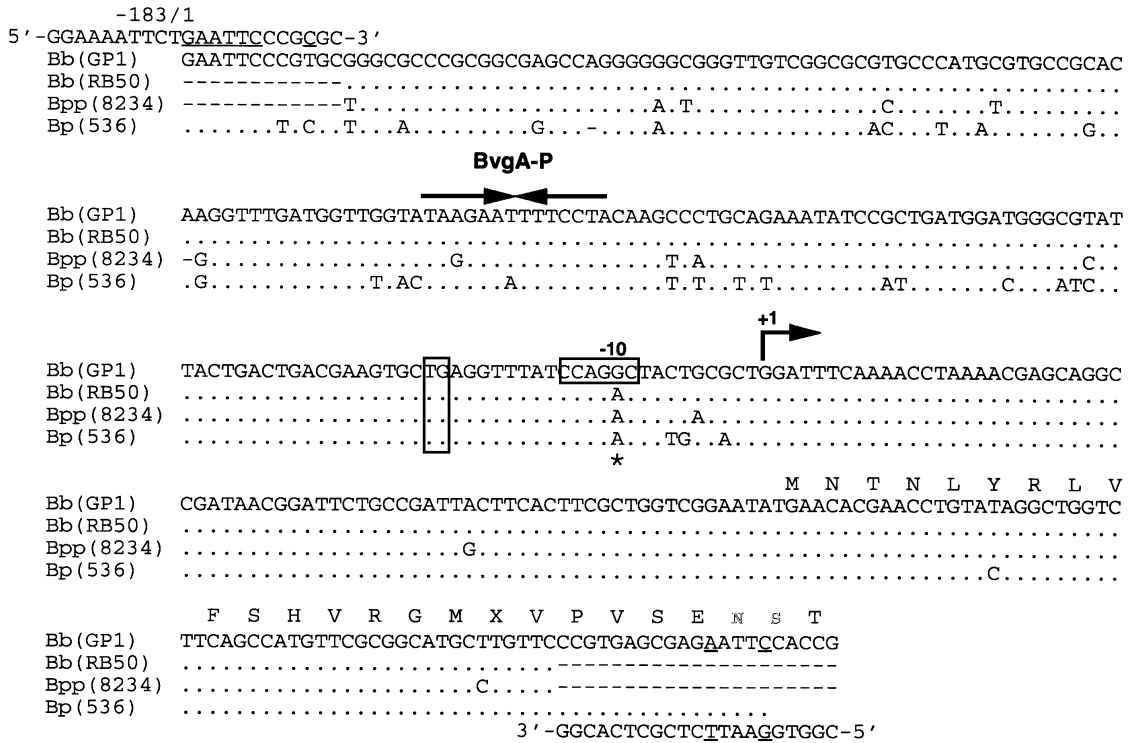


Fig. 3. Comparison of sequences upstream of *fhaB* from various *Bordetella* species. +1 and arrow indicate start of transcription as determined for *B. pertussis* and *B. parapertussis* *fhaB* genes (Roy *et al.*, 1990; Scarlato *et al.*, 1991). A dash indicates no corresponding nucleotide for this position. Boxed regions indicate -10 promoter element and conserved TG located at -25 (Scarlato *et al.*, 1991). An *EcoRI* site was incorporated into the reverse (anti-sense) PCR primer which led to changes in the *fhaB* sequence (underlined) and in the predicted protein sequence (hollow letters). Binding sites for phosphorylated BvgA (BvgA-P) are indicated by arrows (over an imperfect inverted repeat) as shown by gel shifts (Karimova & Ullmann, 1997) and DNase I footprinting (Boucher *et al.*, 1997), X in the protein translation corresponds to L or P in the *fhaB* coding sequences. '*' indicates single base change in GP1 *fhaB* promoter relative to other sequences. Sequences: *B. pertussis* (Bp) 536, GenBank accession no. X152156, *B. bronchiseptica* (Bb) RB50, GenBank accession no. AF111797; GP1, GenBank accession no. AF111796 and *B. parapertussis* (Bpp) 8234, GenBank accession no. AF111798.

indicating that the first residue of FHA Bb is also a modified glutamine, and that the FHA Bb precursor also contains a 71-residue-long signal peptide.

fhaB promoter analysis

Sequence comparison of the ~180 bp promoter/regulatory regions located 5' to the *B. pertussis* and *B. bronchiseptica* *fhaB* genes revealed a number of differences, most of which are located upstream of the transcriptional start site defined for *B. pertussis* *fhaB* 70 nt upstream of the ATG (Roy & Falkow, 1991; Scarlato *et al.*, 1991) (Fig. 3). This region was also amplified by PCR from *B. bronchiseptica* RB50 and *B. parapertussis* 8234, and sequenced. A single nucleotide difference between the *B. bronchiseptica* GP1 (low FHA producer) and the *B. bronchiseptica* RB50 (intermediate FHA producer) promoters was found 11 bases upstream of the transcription initiation site (Fig. 3). The GP1 sequence is unique, with a G at this position; all other *fhaB* promoters analysed contain an A at this position instead. Other substitutions shown in Fig. 3 (e.g. 31 bp differences between GP1 and BP536) distinguish the *B. bronchiseptica* (low or intermediate FHA producers),

B. parapertussis (intermediate FHA producer) and *B. pertussis* (high FHA producer) *fhaB* promoter/regulatory regions.

Two additional sites in the upstream region have been proposed to play important roles in transcriptional control. A TG sequence that has been previously identified (Scarlato *et al.*, 1991) as a common feature of Bvg-regulated promoters is located upstream of the -11 position described above and remains conserved among these *fhaB* sequences. The second regulatory element consists of an imperfect inverted TTTCTA repeat located at position -82 to -95 that is involved in binding of the phosphorylated transcription factor BvgA-P (Boucher *et al.*, 1997; Karimova & Ullmann, 1997). While this element is identical in *B. bronchiseptica* strains GP1 and RB50, there are sequence differences with *B. pertussis* and *B. parapertussis*. Each nucleotide change maintains an imperfect repeat in each *Bordetella* sp., suggesting that this region is unlikely to be responsible for the differences in protein production. In addition, three sites, -15, -16 and -42, have been shown to be responsible for increased *fhaB* expression in the absence of BvgA (Goyard *et al.*, 1995). Interestingly,

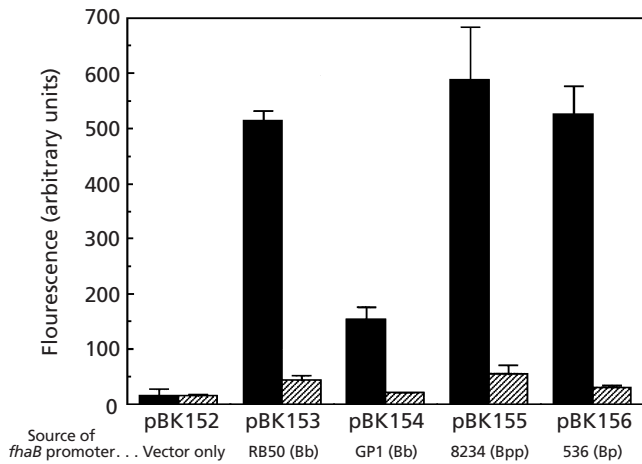


Fig. 4. Fluorescence of promoter-GFP fusion constructs in *B. bronchiseptica* GP1. Fluorescence was detected by FACS analysis of GP1 strains containing fusions of the *fhaB* promoter to *gfpmut3* at 37 °C (black bars) and at 25 °C (hatched bars). Median fluorescence (arbitrary fluorescence units) was measured using four independent transconjugants in at least three independent experiments.

these positions are identical among the four promoter/regulatory regions analysed in this study.

To investigate whether any of these differences might have an effect on promoter activity, the four promoter/regulatory regions shown in Fig. 3 were cloned upstream of a promoterless GFP-encoding sequence. These constructs were introduced into *B. bronchiseptica* GP1 as episomal elements and fluorescence was measured as a marker of *fhaB* promoter activity. *B. pertussis*, *B. paraptussis* and *B. bronchiseptica* RB50 promoters induced similar levels of fluorescence in a Bvg-dependent manner (Fig. 4). In contrast, the fluorescence of the *B. bronchiseptica* GP1 GFP fusion was much lower. These observations indicate that the A at position -11 is important for FHA expression, as the RB50 sequence is otherwise identical to that of GP1 in that region. They also indicate that the nucleotide differences upstream of the transcriptional start site in the three species do not have a net effect on FHA promoter activity; however, their possible effect on transcript stability and processing was not addressed. It is interesting to note that the nucleotide found at the -11 position in nine other Bvg-regulated promoters is also an A (Scarlatto *et al.*, 1991). This nucleotide lies at the fourth position of a sequence in the -10 region, 5'-CAG(A/G)CT-3', which bears some resemblance to the prokaryotic σ^{70} -10 consensus motif, 5'-TATAAT-3'. The expression of the *fhaB* gene is under the control of the two-component system BvgAS, which is down-modulated at low temperatures as well as in the presence of 20 mM MgSO₄. Growth at low temperature (Fig. 4) and in the presence of MgSO₄ (not shown) resulted in the complete absence of fluorescence, indicating that none of the nucleotide substitutions affects BvgA regulation of FHA expression.

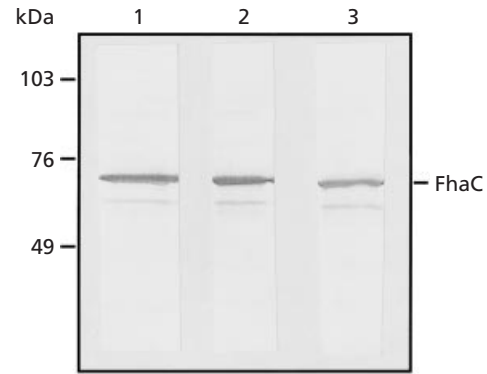


Fig. 5. Production of FhaC in *B. pertussis* BPSM and *B. bronchiseptica* GP1 and BB1015. Total membrane fractions were prepared from exponentially growing cultures of BPSM (lane 1), GP1 (lane 2) and BB1015 (lane 3). Similar amounts of total proteins from the three extracts were analysed by SDS-PAGE followed by immunoblotting with an anti-FhaC antiserum. Molecular masses of size markers are indicated on the left.

The same GFP reporter constructs were also introduced into *B. pertussis* BPGR4 under non-modulating conditions and the results obtained were similar to those measured in GP1 (data not shown). This suggests that the differences in genetic backgrounds of the two species do not grossly affect *fhaB* expression.

Characterization of *B. bronchiseptica* FhaC

The A(-11)G substitution in the *fhaB* promoter region of *B. bronchiseptica* GP1 may account for some of the difference in FHA production between this strain and *B. pertussis*. However, the nucleotide substitutions in the promoter/regulatory regions of the other *B. bronchiseptica* strains and of *B. paraptussis* have no discernible effect on FHA production. In addition, no significant differences were found in the secretion domains of the two proteins, suggesting that the differences in extracellular FHA levels cannot be ascribed to the differences in primary structures of the FHA molecules, and in particular to sequence differences within their secretion domains. We therefore examined the accessory outer-membrane protein FhaC, which is responsible for the extracellular location of FHA in *B. pertussis* (Willems *et al.*, 1994), and the gene that encodes this protein. First, we asked whether FhaC was produced in *B. bronchiseptica* in similar abundance as in *B. pertussis*. Immunoblot analyses were performed on membrane extracts of *B. pertussis* BPSM and *B. bronchiseptica* BB1015 and GP1. Immunoreactive proteins corresponding to FhaC were present in similar quantities in the extracts from the three strains (Fig. 5). Second, the *B. bronchiseptica* *fhaC* gene was amplified from the chromosome of GP1 using the PCR and primers derived from the *B. pertussis* *fhaC* sequence. The amplified fragments were cloned and sequenced on both strands. Translation of the DNA sequence revealed that the *B.*

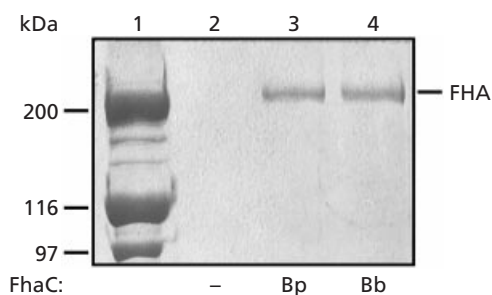


Fig. 6. FHA secretion in BPEC. The *fhaC* mutation of BPEC was complemented by pFJD16 Δ (encoding *B. pertussis* FhaC) or pEC46 (encoding *B. bronchiseptica* GP1 FhaC). Culture supernatants of BPEC (lane 2), BPEC(pFJD16 Δ) (lane 3), or BPEC(pEC46) (lane 4) were analysed by SDS-PAGE and stained with Coomassie brilliant blue.

bronchiseptica GP1 FhaC is 97.4% identical to the published *B. pertussis* FhaC (14 differences and 1 deletion in Gp1).

Although highly similar in their predicted amino acid sequences, we assessed whether the minor differences in the *B. bronchiseptica* FhaC sequence may affect FHA secretion. We therefore compared the level of FHA secretion in *B. pertussis* BPEC, a strain in which the chromosomal *fhaC* gene had been deleted, in the presence or absence of complementation with the *B. pertussis* and *B. bronchiseptica* *fhaC* genes. As shown in Fig. 6, the presence of *B. bronchiseptica* *fhaC* in BPEC restored the secretion of FHA as efficiently as did *B. pertussis* *fhaC*, suggesting that the two encoded proteins are functionally interchangeable and that the 14 amino acid substitutions and one amino acid deletion do not grossly affect the function of FhaC.

DISCUSSION

Mucosal pathogens such as *B. pertussis* and *B. bronchiseptica* rely on multiple attachment factors for colonization of their respective hosts. Both organisms express a similar set of adhesins, the functions of which have been assessed extensively in *B. pertussis*. In this organism, the dominant adhesin is FHA, which is also the most abundant secreted protein. In *B. bronchiseptica*, FHA is required for tracheal colonization of rats, but it is insufficient for colonization when expressed ectopically in the Bvg⁻ phase (Cotter *et al.*, 1998). Most *B. bronchiseptica* isolates produce and secrete FHA at significantly lower levels than *B. pertussis*. This may reflect differences in strength of promoters, in the primary structures of FHA, in the structure or function of the FhaC accessory proteins, or in overall cell-envelope structure. *B. bronchiseptica* and *B. pertussis* synthesize different LPS molecules (Allen *et al.*, 1998), which are likely to confer quite different surface properties on the two species, and therefore possibly to affect FHA secretion or release from the cell surface. However, a *B. bronchiseptica* RB50 derivative with a chromosomal deletion in the locus responsible for O-

antigen synthesis, rendering its LPS more similar to that of *B. pertussis*, did not appear to secrete FHA at higher levels than the parental strain (unpublished results).

The dependence of *B. pertussis* and *B. bronchiseptica* on FHA during early stages of pathogenesis emphasizes the importance of characterizing the relevant genetic loci and their expressed products. As a first step toward the molecular characterization of FHA Bb secretion, we analysed the *B. bronchiseptica* *fhaB* promoter, FHA Bb primary structure and the FhaC Bb accessory protein. Both the structural *fhaB* gene and the *fhaC* gene were found to be highly similar to the *B. pertussis* homologues. Interspecies complementation experiments indicated that the FhaC proteins are interchangeable. In addition, FHA secretion determinants defined in *B. pertussis* were found to be well conserved in *B. bronchiseptica* and they were fully functional.

Downstream of the secretion domain, FHA contains two repeat regions, previously defined as R1 and R2 (Makhov *et al.*, 1994). FHA Bb contains two additional 19-residue repeats in the R1 region. However, this addition did not appear to interfere with the secretion competence or the stability of the protein, as shown by the efficient secretion of *B. bronchiseptica* Fha44, which contains the first 27 of the 40 R1 repeats, in *B. pertussis*. This difference in the number of R1 repeats is therefore not critical for secretion and protein stability, as was also suggested by the isolation of a spontaneous *B. pertussis* Fha44 mutant with a 39-residue deletion corresponding to two repeats in the R1 region. This mutant Fha44 protein remained highly proficient for secretion (our unpublished observation). The R1 repeat region thus tolerates a certain level of variation, possibly indicating some structural plasticity. It should also be noted that both the *B. bronchiseptica* Fha44 homologue and the *B. pertussis* mutant Fha44 were able to bind to heparin-Sepharose as efficiently as *B. pertussis* Fha44, and all three proteins eluted under similar conditions (our unpublished observations). This indicates that the binding site for sulphated sugars, previously mapped in the R1 repeat region (Hannah *et al.*, 1994), is not grossly altered by these structural variations. Other domains with putative or proven roles in FHA-associated adherence functions are predicted to be highly conserved at the level of primary structure in these two species.

The C-terminal domains of the FhaB precursors are highly conserved as well. The function of this domain is not fully understood. A complete deletion of the gene segment corresponding to the 150 kDa C-terminal domain severely hampers FHA secretion in *B. pertussis* (Renauld-Mongenien *et al.*, 1996). However, this region is widely tolerant to smaller deletions (our unpublished observation) with respect to the secretion of FHA. Nevertheless, without creating unmarked *fhaB* chromosomal allelic exchange strains (which presents substantial technical challenges), we cannot fully assess the role of the C-terminal FHA domain in FHA secretion.

An interesting difference between the *B. bronchiseptica* GP1 and the *B. pertussis* BP536 *fhaB* genes lies in their

promoter regions. In the GP1 promoter, a conserved A in the putative -10 motif was replaced by a G. This A to G change was not found in the other *B. bronchiseptica* *fhaB* promoter sequences analysed in the course of this work. Remarkably, an A is invariably found at this position in Bvg-regulated promoters (Scarlatto *et al.*, 1991). The A to G substitution in the GP1 promoter resulted in a significant decrease in *fhaB* expression, as assessed by *gfp* reporter gene fusion experiments, and the decrease in promoter strength is likely to be responsible for the significantly lower FHA production in GP1 compared to other *B. bronchiseptica* strains. It is not known whether this low level of FHA expression reflects an adaptation of the GP1 strain to a particular host or niche and confers some advantage upon this isolate. We cannot rule out the possibility that other interspecies *fhaB* upstream sequence polymorphisms (Fig. 3) might result in altered *fhaB* transcript processing or stability, and explain some of the differences in levels of expressed FHA protein.

The cloning and sequencing of the *B. bronchiseptica* *fhaC* gene revealed that the accessory protein is also very similar to its *B. pertussis* homologue. Complementation of the *B. pertussis* *fhaC* gene by the *B. bronchiseptica* *fhaC* gene resulted in high levels of FHA production and secretion in *B. pertussis*, indicating that the accessory proteins are also functionally interchangeable. However, given our *fhaC*-cloning strategy (see Methods), we can not assess the possible role of the GP1 FhaC G14V substitution in modifying FhaC function. We have also shown that FhaC is produced at levels similar to those in *B. pertussis* in two *B. bronchiseptica* strains which produce and secrete FHA at low and intermediate levels, respectively. Therefore, the primary structure or the amount of the FhaC accessory protein in *B. bronchiseptica* probably does not account for the lower level of FHA secretion by that species.

The function of bacterial adhesins is dictated and regulated at the levels of primary sequence, promoter activity, transcript and protein stability, secretion, and by the context in which adhesins are presented at the bacterial surface. FHA is an important *Bordetella* adherence factor and may play a role in determining host range. We and others have found that FHA is expressed and secreted at different levels by *B. bronchiseptica* and *B. pertussis*. Our data suggest that this variation in FHA secretion/expression may be due to differences in cell-envelope composition, or to differences in *fhaB*-transcript or FHA-protein stability (protein degradation) between the two organisms. These data provide a starting point for further analysis of FHA function and *Bordetella* host species tropism.

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