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

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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.
A noteworthy feature of FHA is its high level of secretion from the rat trachea (Cotter et al., 1992). FHA appears to be produced and secreted by Bordetella pertussis in an 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-Buisne 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).

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-Buisne 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 & Locht, 1992) and *B. pertussis* BP536 (Relman et al., 1989), BPSM (Menozzi et al., 1994a), BPG4R (AfhaB) (Locht et al., 1992) and BPEC (AfhaC) (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 Locht 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 BamHI-restricted pHC79 (Hohn & Collins, 1980), and transformation of *Escherichia coli* DH5α. Cosmid gp1#4 was selected after recognition by colony hybridization with a bgaA gene probe. An EcoRI restriction map suggested that this cosmid also included the fhaB gene. EcoRI restriction fragments of cosmid gp1#4 were subcloned into pBSKII (Stratagene). pBK116 consists of the pBSK1+ vector plus a 10 kb EcoRI 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 2 kb EcoRI fragment from gp1#4 containing the 3′ end of *B. bronchiseptica* fhaB. pBK123 contains a 5 kb BamHI insert corresponding to the 3′ half of the 10 kb EcoRI 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 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 gfpmut3 fragment from an EcoRI/HindIII digest of pGFPmut3 into pBRR1MC5 (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′-GGAAAATTCTAGAATTCCCG- CGC-3′) and FHA328RR (5′-CGGTGAAATTCCTCCTCCT- CCG-3′). The EcoRI 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 EcoRI site. The PCR was performed using

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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.
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 insert 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–BamHI 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 pBBRI122, a vector that is able to replicate in Bordetella spp. (Antoine & Locht, 1992). The internal 71 kb BamHI fragment was then deleted by digesting the resulting plasmid with BamHI and re-ligating it, thereby yielding pEC40.

pFJD16Δ was obtained as follows. The unique PstI site of pBBR1MCS was removed using a T4 polymerase treatment of the PvuII-restricted plasmid, followed by re-ligation. The 2.2 kb SalI–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 PstI–SacI 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 Rp, 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 Quagen Genomic DNA kit, using the oligonucleotides 5′-ATGACTGACGCAACGAACCGTTT-3′ and 5′-GCGTTCTCGCGCGCTAGAACGT-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 A450 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, uncentred 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...
Nevertheless, BPSM, whereas the amount of FHA in GP1 supernatants reproducibly four- to eightfold lower than those of BB1015 (lanes 3) and of FHA secretion by B. pertussis. Immunoblot analysis of cell extracts and by the haemagglutination test showed that FHA in all four isolates roughly paralleled those of FHA Bp shown. Interestingly, the amounts of cell-associated FHA was obtained from GP1 culture supernatants released in the culture supernatants, as assessed 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.

**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.

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 test 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 BPSM36 (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 (RLRSRIARTTGGSMKPTNR) should be replaced beginning at residue 3571 with RLRVED-IGKKNYRFFYETNK, 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. parapertussis 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. parapertussis 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. parapertussis, 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).
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 is also blocked. In the first few cycles of secretion, minute chromatographic peaks corresponding to the QGLVP sequence were detectable (data not shown), suggesting that the N-terminal residue of the FHA Bb, we overproduced a truncated, N-terminal portion of the GP1 FHA in B. bronchiseptica. This protein, which is the B. bronchiseptica equivalent of Fha44 (Renauld-Mongenie 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),
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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 fli10 promoter element and conserved TG located at fli25 (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 producer), 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 TTTCCTA repeat located at position ~25 to ~95 that is involved in binding of the phosphorylated transcription factor BvgA (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,
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. parapertussis* 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 (Scarlato 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 σ70 −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.

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. parapertussis* 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.
bbronchiseptica 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 genic 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-Mongenie 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 (Scarlato 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 fhaC gene revealed that the accessory protein is also very long-lived. FHA is an important virulence determinant of *Bordetella* spp. FHA is a filamentous hemagglutinin (FHA) and is expressed at levels similar to those in *B. pertussis* in two *B. bronchiseptica* strains producing 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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