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Bordetella bronchiseptica Expresses the Fimbrial Structural Subunit Gene *fimA*

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The differential host species specificities of *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica* might be explained by polymorphisms in adherence factor genes. We have found that *B. parapertussis* and *B. bronchiseptica*, unlike *B. pertussis*, contain a full-length gene for the fimbrial subunit FimA. *B. bronchiseptica* expresses *fimA* in a BvgAS-dependent fashion.

The genetic basis for fimbrial expression in *Bordetella pertussis* has been well characterized. A fimbrial operon located downstream of the filamentous hemagglutinin structural gene *fhaB* is regulated by the BvgAS two-component system; it contains genes encoding accessory proteins (FimB and FimC) and the fimbrial minor subunit (FimD) (18). The genes for the major fimbrial subunits, Fim2 and Fim3, are expressed elsewhere on the *Bordetella* chromosome (8, 10). Fimbrial phase variation can be observed in vivo and is controlled by small insertions or deletions in a C-rich region upstream of both *fim2* and *fim3* (17). The *B. pertussis* fimbrial operon also contains a pseudogene designated *fimA*, located at the 5′ end of the fimbrial gene cluster (Fig. 1) (18). This gene contains a DNA sequence homologous to those of *fim2* and *fim3* but lacks sequences predicted to encode the N-terminal third of the fimbrial subunit (18).

The organization of the *Bordetella bronchiseptica* and *Bordetella parapertussis* fimbrial genes and their function are less well characterized than those of the *B. pertussis* fimbrial genes (5, 6). *B. bronchiseptica* expresses proteins that are recognized by polyclonal and monoclonal antisera generated against *B. pertussis* Fim2 and Fim3 (11, 16). Coding sequences for Fim2 and Fim3 are 74 and 94% similar at the nucleotide level, respectively, between *B. pertussis* and *B. bronchiseptica* (16). Comparison of 5′ sequences upstream of *fim2* and *fim3* in the two species suggests similar mechanisms of transcriptional control. Accessory fimbrial proteins in *B. bronchiseptica* have not been examined in any detail, although the minor fimbrial subunit in this species, FimD, is predicted to differ from that of *B. pertussis* by only one amino acid (19). Differences in the host species specificities of these three *Bordetella* species might reflect polymorphisms at the genetic loci that encode fimbriae and other adherence factors.

Structure of *B. bronchiseptica* and *B. parapertussis* genomic regions encompassing *fimA*. Using PCR primers derived from previously published *B. pertussis fhaB* (positions 10756 to 10781; GenBank no. X52156) and *fimA* (746 to 729; GenBank no. X64876) sequences (4, 18), we detected a 400-bp amplicon size polymorphism between *B. pertussis* BP536 (13) and *B. bronchiseptica* GP1, RB50, 110H, B133, and VPI-FE1 (2, 3) that corresponded to the region just downstream of *fhaB*. (Sequence analysis at the 3′ end of GP1 *fhaB* revealed a substitution, G10781T, at the 3′ end of the forward priming site.) The 1,105-bp amplicon from *B. bronchiseptica* guinea pig isolate GP1 (1) was cloned in pBluescript II KS (Stratagene, La Jolla, Calif.), and its sequence was determined with the PRISM Ready Reaction Dye Deoxy Terminator Cycle sequencing kit (Perkin-Elmer) and a 373A automated DNA sequencer (Applied Biosystems Inc., Foster City, Calif.). ULTma DNA polymerase (Perkin-Elmer), with 3′-5′ exonuclease proofreading activity, was used to amplify DNA for cloning, to minimize *Taq*-associated errors. All PCR mixtures contained 2.5% formamide. The corresponding chromosomal regions from *B. bronchiseptica* porcine isolate B15 (W. Gaastra, University of Utrecht, Utrecht, The Netherlands) and *B. parapertussis* human isolate B24 and ovine isolates 9400142 and 9300379 (J. F. Porter, Moredun Research Institute, Edinburgh, Scotland) were also sequenced.

GP1 genomic sequence analysis revealed a complete *fimA* gene (606 bp) beginning approximately 330 bp from the end of the *fhaB* open reading frame and a segment comprising 419 bp beginning 65 bp from the end of *fhaB* that is not found at the corresponding site in the *B. pertussis* chromosome (Fig. 1). Within the GP1 419-bp insert, there is a putative BvgA binding site, TTTCCTA (14), and a putative −10 region. There is no apparent "C stretch," which has been found upstream of *fim2*, *fim3*, and *fimX* open reading frames in *B. pertussis* and *B. bronchiseptica* and which is believed to be involved in fimbrial phase transitions (16, 17). At nucleotide position 393 of the *B. bronchiseptica fimA* open reading frame there is a 9-bp insertion relative to the truncated *B. pertussis* gene. The *fhaB-fimA* intergenic regions of the *B. bronchiseptica* B15 porcine isolate and GP1 are nearly identical (only 12 nucleotide differences over approximately 1,050 bp).

All three *B. parapertussis* strains contained the 419-bp insert and a complete copy of *fimA*. Of note, the *B. parapertussis* B24 human isolate carries a frameshift mutation (insertion of a G) in the 10th codon of *fimA* that is not found in either of the *B. parapertussis* ovine isolates (9400142 and 9300379).

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FIG. 1. Schematic diagram of *B. pertussis* and *B. bronchiseptica* genomic structure at the *fimA* region. Directional boxes indicate open reading frames. The additional DNA found in *B. bronchiseptica* beginning upstream of *fimA* and including the 5′ end of *fimA* is depicted by diverging dotted lines and a shaded box. Arrowheads indicate the location of the PCR primers used for amplification of the *fimA*-containing region. The *fimA Sal*I sites used for reporter and deletion construction are indicated.

Predicted FimA amino acid sequences and phylogeny. The complete *fimA* gene in *B. bronchiseptica* and *B. parapertussis* is predicted to encode a 201-amino-acid protein (Fig. 2). There is one amino acid difference between the predicted sequences from *B. bronchiseptica* strains GP1 and B15, and there are two positions of variation among the sequences of the three *B. parapertussis* strains. The C-terminal portion of the predicted FimA sequence that is encoded within the *B. pertussis* chromosome is ~95% identical to the corresponding region of the predicted *B. bronchiseptica* FimA sequence. All available complete *Bordetella* sp. fimbrial subunit gene sequences were aligned, and evolutionary relationships were inferred by using parsimony methods and a masked data set comprising only those positions for which information was available for all sequences (570 positions) (8, 10, 12, 16, 18). This analysis revealed closer relationships between *fimA* and *fim3* homologs than between *fimA* and either *fim2* or *fimX* (data not shown). It also suggests that the different types of fimbrial subunit genes diverged prior to the divergence of the different *Bordetella* species.

***fimA* expression.** To examine whether the full-length *fimA* gene in *B. bronchiseptica* is expressed, we cloned an internal 288-bp *Sal*I fragment of the GP1 *fimA* gene upstream of the promoterless *lacZ* gene in pEGHZ3 (gift from P. Cotter and J. F. Miller, University of California, Los Angeles) and introduced this reporter construct into the chromosomes of GP1 and the *B. bronchiseptica* rabbit isolate, RB50 (3), by homologous recombination, creating the *fimA*::*lacZ* chromosomal fusion strains GPFF96 and RBFF96. As a control, the *fimA* fragment was cloned in the reverse orientation, creating strains GPRF96 and RBRF96. Proper chromosomal integration of the plasmids was confirmed by Southern analysis. Expression of *lacZ*, and thus *fimA*, was determined by measuring β-galactosidase activity in *B. bronchiseptica* mid-log-phase cultures grown in Luria-Bertani broth as previously described (15). GPFF96 and RBFF96 produced 54- and 52-fold more β-galactosidase activity than GPRF96 and RBRF96 ($P = 0.017$ and 0.005, paired t test), respectively (Fig. 3). We also performed the same experiment under conditions known to cause BvgAS modulation (20 mM $MgSO_4$). Under these conditions, *fimA* expression in GPFF96 and RBFF96 was reduced to approximately the levels observed in GPRF96 and RBRF96. The difference in the β-galactosidase activities of GPFF96 under nonmodulating and modulating conditions was statistically significant ($P = 0.019$), as were those of RBFF96 ($P = 0.004$, paired t test). These results suggest that expression of *fimA* of *B. bronchiseptica* is regulated by BvgAS.

Expression of FimA was also assessed by means of Western immunoblot analysis. A chromosomal in-frame *fimA* deletion was constructed in GP1 and RB50 by deleting the 288-bp *Sal*I fragment from the cloned *fimA* gene and exchanging the resulting deletion allele for the wild-type copy by using the pEG7 suicide vector (gift from P. Cotter) and *sacB*-encoded sucrose sensitivity for counterselection against merodiploids (3). The expected deletion mutations were confirmed by Southern analysis of the two resulting strains, GP96 and RB96. We examined whole-cell lysates from a standardized number of BP536, GP1, RB50, GP96, and RB96 cells, by separating proteins electrophoretically on a polyacrylamide gel, transferring them to ni-

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           1                                                                                                     100
   BpFim2  MQIPFQRALR LCLRAALAAI ASAAHADDGT IVITGTITDT TCVIEDPSGP NHTKVVQLPK ISKNALKANG DQAGRTPFII KLKDCPSSLG N.GVKAYFEP
   BbFim2  MQVPFQRALP LCLRAALAAI ASAAHADDGT IVITGTITDT TCVIEDPAGP THTKVVQLPK ISKSALAKDG DEAGRTPFLI TLKDCPSSLN N.GVKAYFEP
   BpFimX       MQAKT FLLGAALAGV ALAAHAEDGT IVITGTITDQ TCTIEDPS.P GYIKVVHLPT ISKSALKNAG DVAGRTRFDI KLKDCPTTVN ..TLKLYFEP
   BpFim3  MSKFSYPALR AALILA.ASP VLPALANDGT IVITGSISDQ TCVIEEPSTL NHIKVVQLPK ISKNALRNDG DTAGATPFDI KLKECPQALG ..ALKLYFEP
   BbFim3  MSKFSYPALR TALILA.ASP VLPAHANDGT IVITGSISDQ TCVIEEPSAP NHIKVVQLPK ISKNALRNDG DTAGATPFDI RLKECPQ.LG ..ALKLYFEP
   BpFimA                                                     IAVQLPT ISRTALKDVG STAGGTVFDV KLTECPQALN GQQVGLFFES
 BppFimA    MNLKFAGIA LGLTACALTY QHQVFAADGT LVITGAITDT TCKINGAE.P PTNITVQLPT ISRTALKDVG STAGGTVFDV KLTECPQALN GQQVGLFFEP
   BbFimA    MNLKFAGIA LGLTACALTY QHQVFAADGT LVITGAITDT TCKINGAE.P PTNIAVQLPT ISRTALKDVG STAGGTVFDV KLTECPQALN GQQVGLFFEP
                                  . * *** :****:*:*  ** *: .      .*:**. **:.**   * . ** * * :  *.:**  :.    :  :**.

           101                                                                                                   200
   BpFim2  GPTTDYSTGD LRAYKMVYAT NPQTQLSNIT AATEAQGVQV RISNLNDSKI TMGANEATQQ AAGFDPEVQT GG..TSRTVT MRYLASYVKK N.GDVEASAI
   BbFim2  GPTTDYATGD LKAYSIAYNN NPATTQNAII AASEAQGVQI RISNQNGTKI PMGVDAAAQN AQAFNPVTDT ADN.AKKKVT LRYLASYVKK S.GNITAGQL
   BpFimX  GPTTDYGTKD LKAYKQAWYV DAATLLKSPP SVTEAKGVQI RLMNLNGKQI PMGETEPNQH AAAFSGTMQA GQ..GQKSFT LHYLAGYVKK ASGEVEATML
   BpFim3  GITTNYDTGD LIAYKQTYNA SGNGNLSTVS SATKAKGVEF RLANLNGQHI RMGTDKTTQA AQTFTG.KVT NG...SKSYT LRYLASYVKK PKEDVDAAQI
   BbFim3  GITTNYDTGD LIAYKQAYNA SGNGNLSTVS SATKAKGVEF RLANLNGQHI RMGTDETTQA AQTFTGTDVT NGGNTTKSYT LRYLASYVKK PNEDVDAAQM
   BpFimA  GGTVDYTSGN LFAYR..... .ADSQGVEQV PQTKADNVQ. ..ANLDGSAI HLGRNKGAQA AQTFLVSQTA GS..STYGAT LRYLACYIRS GAGSIVAGNL
 BppFimA  GGTVDYTSGN LFAYR..... .ADSQGVEQV PQTKADNVQF QLANLDGSAI HLGRNEGAQA AQTFLVSQTA GS..STYGTT LRYLARYIRS GAGSIVAGNL
   BbFimA  GGTVDYTSGN LFAYR..... .ADSQGVEQV PQTKADNVQF QLANLDGSAI HLGRNKGAQS AQTFLVSQTA GS..STYGTT LRYLARYIRS GAGSIVAGNL
           * *.:* : : * **            . ::*..*:    *:.   *  :*     *  *  *     :          * ::*** *::.    .: *  :

           201      211
   BpFim2  TTYYGFSVVY P
   BbFim2  TTYVGFSMIY P
   BpFimX  TTYVGFSVVY P
   BpFim3  TSYVGFSVVY P
   BbFim3  TSYVGFSVVY P
   BpFimA  RSQVGFSVMY P
 BppFimA  RSQVGFSVMY P
   BbFimA  RSQVGFSVMY P
            :  ***  * *
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FIG. 2. Alignment of predicted amino acid sequences for *B. pertussis* (Bp) truncated FimA (18), *B. parapertussis* (Bpp) B24 FimA, *B. bronchiseptica* (Bb) GP1 FimA, *B. bronchiseptica* 685 Fim2 (16), *B. bronchiseptica* 685 Fim3 (16), and *B. pertussis* Fim2 (8), Fim3 (10), and FimX (12). The *B. parapertussis* B24 FimA sequence was generated by deleting the single nucleotide insertion found in the 10th codon. Dots within the alignment indicate spaces that were added to increase the number of residue matches. Below the sequences, asterisks indicate positions with identical residues in all sequences, colons indicate positions with closely related residues in all sequences, and dots indicate positions with less closely related but similar residues in all sequences.

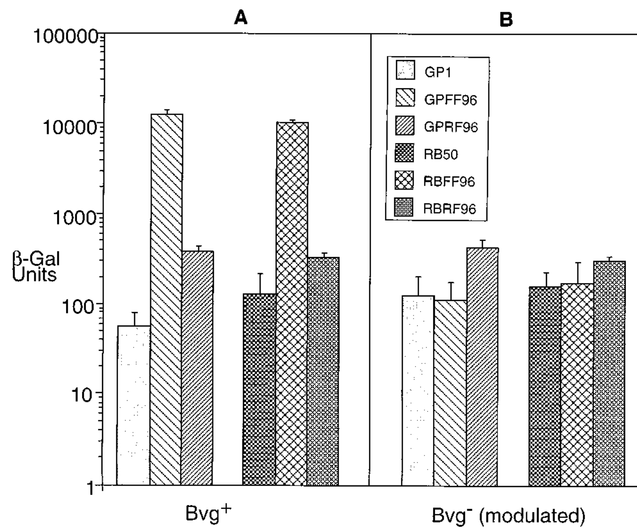

FIG. 3. Production of β-galactosidase (β-Gal) by *B. bronchiseptica* GP1 and RB50 *fimA*::*lacZ* fusion strains under nonmodulating (Bvg$^+$ [A]) and modulating (Bvg$^-$ [B]) conditions. Strains GPFF96 and RBFF96 contain the *fimA*::*lacZ* fusion in the forward orientation, while GPRF96 and RBRF96 contain the fusion in the reverse orientation. Bars indicate the standard errors of the means in three independent experiments.

trocellulose, and probing them with polyclonal antiserum directed against *B. pertussis* Fim2 (11). This antiserum is known to cross-react with other *Bordetella* fimbrial subunit proteins. These lysates all contained a polypeptide of a size consistent with that of *B. pertussis* and *B. bronchiseptica* Fim2 (22 to 23 kDa) which reacted with this antiserum (Fig. 4). However, the GP1 (Fig. 4, lane 2) and RB50 (data not shown) lysates also contained a second, fainter polypeptide band, approximately 1 kDa smaller than Fim2, which also reacted with the Fim2 antiserum. GP96 (Fig. 4, lane 3) and RB96 (data not shown), with chromosomal *fimA* deletions, lacked this second polypeptide band, suggesting that this smaller cross-reactive protein is FimA and that it is expressed in *B. bronchiseptica.*

We have shown that *B. bronchiseptica* contains an intact *fimA* gene which is expressed in a BvgAS-regulated manner and is similar to *B. pertussis fimA*, *fim2*, *fim3*, and *fimX* at both the nucleic acid and amino acid levels (8, 10, 12, 18). In addition, an in-frame *B. bronchiseptica fimA* knockout mutant lacks a polypeptide present in wild-type GP1 that cross-reacts with *B. pertussis* Fim2 antiserum and corresponds in size to the predicted size of FimA, suggesting that *B. bronchiseptica* FimA

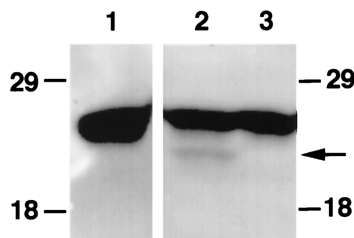

FIG. 4. Western immunoblot of *Bordetella* protein lysates obtained with polyclonal anti-*B. pertussis* Fim2 antisera. Lane 1, *B. pertussis* BP536; lane 2, *B. bronchiseptica* GP1; lane 3, GP1 *fimA* deletion mutant GP96. A polypeptide of approximately 22 kDa (Fim2) from all three strains reacts with these cross-reacting antisera, as expected. In addition, a polypeptide approximately 1 kDa smaller (arrow) is expressed by GP1; this smaller polypeptide is not expressed by the GP1 *fimA* deletion mutant. Numbers are molecular masses, in kilodaltons.

is expressed. The hypothesis that *B. bronchiseptica fimA* is expressed is supported by a study that shows that *B. bronchiseptica* expresses one more major fimbrial protein than *B. pertussis* (7). Additional evidence for *B. bronchiseptica* FimA expression derives from N-terminal amino acid sequence analysis of *B. bronchiseptica* fimbrial subunits that cross-reacted with *B. pertussis* Fim2 antiserum (11). All three analyzed subunits, isolated from different *B. bronchiseptica* strains, revealed an N-terminal sequence that was essentially identical to the sequence predicted by the *fimA* gene sequence. Furthermore, the three analyzed subunits migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis at a position similar to that of the protein affected by the knockout mutation in strains GP1 and RB50 in this study, i.e., slightly faster than Fim2.

The *fimA* gene is located at a genomic position where major fimbrial subunit genes are generally found in other fimbrial gene clusters (9), suggesting that it may be the present-day derivative of the primordial major subunit gene. Thus, it is conceivable that *fim2*, *fim3*, and *fimX* are derived from an ancestor of *fimA* that was duplicated to positions outside the *fim* gene cluster. The position of the cluster adjacent to *bvgAS* and *fhaB*, the structural gene for filamentous hemagglutinin, which is the major adhesin for the *Bordetella* species, is consistent with this hypothesis. An interesting feature of the *B. bronchiseptica fimA* DNA sequence is that it lacks the string of cytosine residues present upstream of the major *B. pertussis* fimbrial subunits (17). This suggests that *B. bronchiseptica fimA* may not undergo phase variation in the same manner as do the major *B. pertussis* fimbrial subunits.

Some evidence suggests that *B. bronchiseptica* fimbrial expression might contribute to host species specificity (2). Thus, it is tempting to speculate that the difference in host range between *B. bronchiseptica* and *B. pertussis* is at least partially due to differences in *fimA* expression. It is interesting that *fimA* is inactive in all *Bordetella* species and strains that are commonly isolated from humans. Although the function of *fimA* has not been discerned, characterization of *fimA* genes may contribute to a more complete understanding of fimbrial subunit gene evolution and host adaptation in the genus *Bordetella.*

Nucleotide sequence accession numbers. The DNA sequences for the *fimA* regions of *B. bronchiseptica* GP1 and B15 and of *B. parapertussis* B24, 9400142, and 9300379 were assigned GenBank accession numbers AF022303 to AF022307, respectively.

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