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Common accessory genes for the *Bordetella pertussis* filamentous hemagglutinin and fimbriae share sequence similarities with the *papC* and *papD* gene families

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The *Bordetella pertussis* filamentous hemagglutinin (FHA) is a major virulence factor responsible for attachment, one of the early events in bacterial pathogenesis. Deletion of its structural gene, *fhaB*, or a Tn*5* insertion in *fhaA*, downstream of *fhaB*, resulted in a FHA$^-$ and fimbriae$^-$ phenotype, although *fhaB* and the *fim* genes are not linked. The *fhaB* downstream region therefore most likely encodes accessory proteins required for the biosynthesis of FHA and fimbriae, despite the lack of sequence similarities between these two proteins. The nucleotide sequence of this area contains the open reading frames *fhaD* and *fhaA*, whose products share sequence similarities with the *papD* and *papC* gene products, respectively. PapD is a periplasmic chaperone protein able to bind to the *Escherichia coli* P pilin subunits and to transport them towards the outer membrane protein PapC which is responsible for pilus membrane translocation. An additional open reading frame, *fhaE*, is located downstream of *fhaA*. Its amino acid sequence shares similarities with those of the fimbrial subunits. Deletion analyses suggest that *fhaB* and the downstream genes can be transcribed as a polycistronic operon, and primer extension analysis revealed the presence of a second promoter between *fhaB* and *fhaD*.

Key words: assembly/chaperone/pili/secretion/virulence factor

Introduction

Specific attachment of pathogenic organisms to the host tissues is one of the first events in the pathogenesis of infectious diseases. For many Gram-negative bacterial pathogens, this step is mediated by filamentous structures called pili or fimbriae (Duguid and Old, 1980). *Bordetella pertussis*, the etiologic agent of whooping cough, produces at least two different filamentous structures at its surface, fimbriae and filamentous hemagglutinin (FHA), in addition to at least two other attachment factors, pertussis toxin (PTX) and pertactin (Weiss and Hewlett, 1986). All these virulence factors are coordinately regulated at the transcriptional level by the gene products of a central regulon named *vir* or *bvg* (Arico *et al.*, 1989).

Whereas the involvement of the *B.pertussis* fimbriae in attachment and colonization is not yet conclusively established (Robinson *et al.*, 1990), FHA has been clearly shown by several laboratories to mediate attachment of the pathogen to the epithelium of the upper respiratory tract (Tuomanen and Weiss, 1985; Relman *et al.*, 1989; Kimura *et al.*, 1990). At least three different binding activities for cellular attachment of FHA have been identified thus far. The best studied mechanism involves one of the two RGD sequences found in the FHA sequence (Relman *et al.*, 1989). FHA may bind to integrins on pulmonary macrophages via this RGD sequence (Relman *et al.*, 1990). This binding activity can be inhibited by specific peptides containing the RGD sequence. Furthermore, binding of FHA to epithelial cells can be inhibited by the addition of galactose (Tuomanen *et al.*, 1988), indicating the existence of a lectin-like binding site on the FHA molecule. Recently, we found that FHA can also specifically bind to sulfated saccharides such as heparin (Menozzi *et al.*, 1991), which may be the mechanism of the binding of *B.pertussis* to sulfated glycolipids (Brennan *et al.*, 1991). These lipids are present in large amounts in the human trachea and lung, and have been identified as receptors for other respiratory pathogens (Krivan *et al.*, 1989).

The structural gene encoding FHA (*fhaB*) has been cloned (Mattei *et al.*, 1986; Brown and Parker, 1987; Stibitz *et al.*, 1988; Relman *et al.*, 1989; Delisse-Gathoye *et al.*, 1990) and was shown to contain an unusually large open reading frame (ORF) with a coding potential for a 370 kDa protein, whereas extracellular FHA has an apparent molecular mass of 220 000 (Delisse-Gathoye *et al.*, 1990; Domenighini *et al.*, 1990). Important post-translational maturation thus occurs during the biosynthesis of this protein. Immunological analyses have shown that the mature FHA contains the N-terminal two-thirds of the precursor molecule, and that the C-terminal end is removed during biosynthesis (Delisse-Gathoye *et al.*, 1990). In addition, large amounts of FHA are secreted into the culture medium, although the protein can also be found attached to *B.pertussis* cells. The organism therefore possesses a very efficient outer membrane translocation mechanism that most likely requires the expression of FHA accessory genes.

In this study, we have identified accessory genes involved in the biosynthesis of FHA and show that their gene products share sequence similarities with the PapD and PapC protein families that are essential for the biosynthesis of pili in Gram-negative organisms. In addition, mutations in the FHA accessory genes also abolish the production of the *B.pertussis* fimbriae, indicating that the same accessory genes are involved in the production of the two different filamentous adhesive structures in *B.pertussis*.

Results

Deletion of fhaB from the B.pertussis chromosome

To gain insight into the mechanism of FHA biogenesis, we first deleted its structural gene from the *B.pertussis*

chromosome by two successive homologous recombination events using pMC2. This plasmid is a pSS1129 derivative containing the upstream and downstream flanking regions of *fhaB*, connected by part of the pUC18 multiple cloning site as shown in Figure 1.

Western blot analysis of the *fhaB* deletion mutant *B.pertussis* BPMC using anti-FHA monoclonal antibodies showed that this strain does not produce any detectable FHA, in neither a secreted, nor a cell-associated form (Figure 2A).

Since one of the homologous recombination events that led to the deletion of *fhaB* involved the *bvg* gene, responsible for the production of all the known *B.pertussis* virulence factors, it was of importance to investigate whether *B.pertussis* BPMC is also affected in the expression of other virulence genes. No difference was observed between *B.pertussis* BPMC and the Tohama I parent strain with respect to the production of hemolysin and PTX as evidenced by hemolysis and Western blot analyses using anti-PTX monoclonal antibodies. These results indicate that the *fhaB* deletion did not abolish the *bvg* function and that the *fhaB* upstream region is unaffected in *B.pertussis* BPMC.

Western blot analysis using anti-fimbriae monoclonal

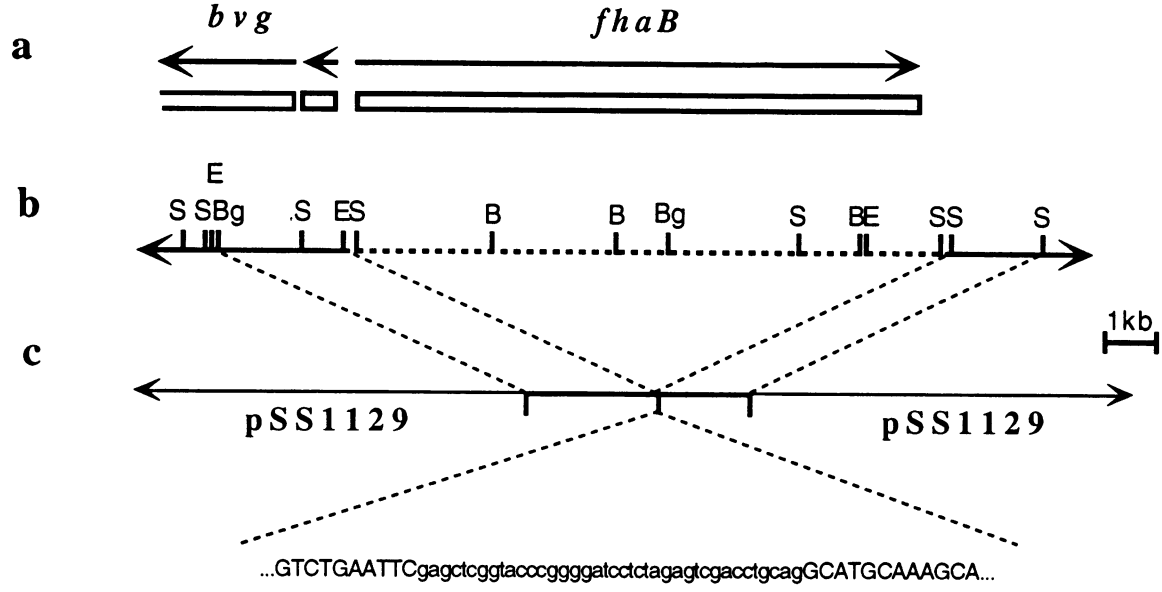


Fig. 1. Deletion of the *fhaB* gene. (**a**) The *fhaB* and the bicistronic *bvg* genes are indicated by the boxes representing the respective ORFs, and the arrows showing the direction of their translation. (**b**) The *B.pertussis fhaB* locus. The black line represents the DNA remaining in *B.pertussis* BPMC, and the dotted line indicates the DNA fragment deleted in this strain. The arrows point to the rest of the chromosomal DNA. Restriction sites shown are B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI and S, *Sph*I. (**c**) pMC2 used for the deletion of *fhaB*. The thick line represents *B.pertussis* DNA (the *fhaB* flanking regions) and the thin line shows pSS1129 plasmid DNA. The nucleotide sequence between *Eco*RI and *Sph*I in pMC2, as well as in *B.pertussis* BPMC, is shown at the bottom. Upper-case letters show the *B.pertussis* DNA sequence and lower-case letters show the pUC-derived nucleotide sequence.

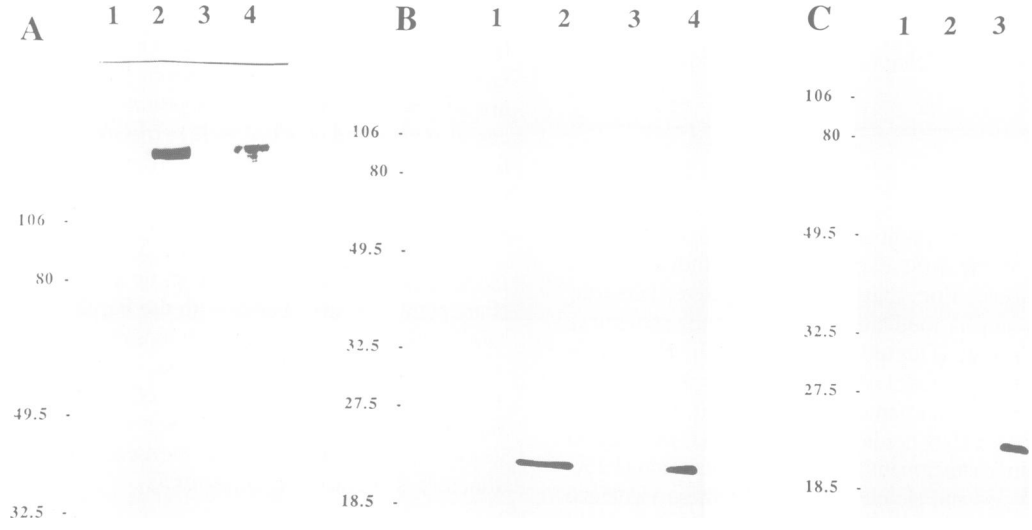


Fig. 2. Western blot analysis of *B.pertussis* BPMC, Tohama I and BP353. Western blot analysis was performed using anti-FHA monoclonal antibody 12.1F9 (**A**) or anti-fimbriae monoclonal antibody BPA10 (**B**) on culture supernatants (A and B, lanes 1 and 2) or total cell extracts (A and B, lanes 3 and 4). The production and secretion of FHA and fimbriae in *B.pertussis* BPMC (A and B, lanes 1 and 3) were compared with those of the Tohama I control strain (A and B, lanes 2 and 4). The production of fimbrial subunits was also analyzed on *B.pertussis* BP353 (C, lane 1), and compared with BPMC (C, lane 2) or Tohama I (C, lane 3). The molecular mass markers expressed in kDa are given in the margins.

antibodies also revealed that *B.pertussis* BPMC does not produce any detectable fimbrial subunits (Figure 2B). The absence of fimbriae from this strain was surprising, since the structural genes of the fimbrial subunits are not closely linked to the *fhaB* gene (Willems *et al.*, 1990). It is therefore likely that the *fhaB* deletion affected the expression of downstream genes that are somehow involved in fimbrial biosynthesis.

Nucleotide sequence analysis of the fhaB downstream region

The nucleotide sequence of the DNA fragment located between the stop codon of the *fhaB* ORF and the *Pst*I site located 5.4 kb downstream was determined in both directions. The 5′-end of the sequence overlaps 802 bp of the 3′-end of the DNA fragment sequenced by Domenighini *et al.* (1990) with the following differences: the deletion of a C at position 276, the deletion of a G at position 285, the insertion of a C at position 701, the change from G to C at position 772, the insertion of GGC at positions 777–779, and the insertion of CGCCG at positions 799–803 (the numbers refer to the nucleotide positions after the TAG stop codon of *fhaB*).

The sequenced region spanning 5440 bp has a G+C content of 66.5%, very similar to the composition of other *B.pertussis* virulence genes (Locht and Keith, 1986; Glaser *et al.*, 1988; Domenighini *et al.*, 1990). No sequence similarity was detected between this DNA fragment and other sequences in the EMBL data library. When the DNA sequence was scanned for the protein coding regions, three ORFs were found oriented in the same direction as *fhaB*. Computer analysis using the CODONPREFERENCE program indicated that all three have a high coding probability. The first ORF extends from nucleotide position 758 to 1489 and contains 244 codons. The second ORF extends from position 1555 to position 4173 and contains 873 codons, and the third ORF from position 4157 to position 5284 and contains 376 codons. The second and third ORFs overlap by 17 nucleotides. All ORFs start with a proposed ATG initiation codon and end with a TGA stop codon. The initiation codon of the first ORF is located downstream of a sequence homologous to *Escherichia coli* ribosomal binding sites. No such consensus sequence could be found upstream of the two other ORFs. No promoter consensus sequence was identified that resembled either *E.coli* promoter sequences, or the promoters of *fhaB* or the pertussis toxin gene (Locht and Cabezon, 1990). However, the first 700 bp upstream of the first ORF are characterized by a relatively high A+T content of 41.14% (with 22.71% T in the coding strand). The promoter region of the *bvg* gene also contains stretches relatively rich in T.

The second ORF is located in the region disrupted by a Tn*5* insertion in *B.pertussis* BP353 (Figure 4), a strain characterized by a defect in FHA production (Weiss *et al.*, 1983). This strain produces very small amounts of cell-associated FHA (unpublished observation). We have confirmed the location of the Tn*5* insertion by DNA sequencing after subcloning of the Tn*5* flanking region from *B.pertussis* BP353. The transposon insertion was found to be located at position 2149. The gene inactivated by the transposon was previously named *fhaA* (Stibitz *et al.*, 1988). Therefore, the second ORF corresponds to the sequence of *fhaA*. Since a *fhaC* gene has been mapped elsewhere (Stibitz *et al.*, 1988), we have consequently named the first and third ORFs reported in this study *fhaD* and *fhaE*, respectively.

Amino acid sequence analysis of FhaA and FhaD and FhaE

The proteins encoded by *fhaD*, *fhaA* and *fhaE* begin with amino acid sequences that strongly resemble typical prokaryotic signal peptides, suggesting that they cross the inner membrane of the organism. The calculated molecular weights of the mature FhaD, FhaA and FhaE proteins are 23 647, 91 012 and 36 705, respectively. Their calculated isoelectric points are 6.63, 9.55 and 7.13 respectively. The charges of FhaD are uniformly distributed throughout the protein, whereas the charged residues in FhaA and FhaE are predominantly found in the N-terminal portions of the molecules. Hydrophobicity analysis of FhaA revealed no major hydrophobic region of 15 consecutive amino acids or more.

When the amino acid sequences of the ORFs were scanned against the EMBL data bank, striking sequence similarities were detected between FhaD and FhaA, and the proteins encoded by the *papD* and *papC* gene families, respectively. The *papD* and *papC* genes encode a chaperone and an outer membrane protein, respectively, both essential for the biogenesis of the *E.coli* P pilus (for a recent review, see Hultgren *et al.*, 1991).

The proteins encoded by *fhaD* and by *papD* share 33.2% amino acid identity. Seven percent of the amino acid residues are conserved among all members of the PapD family (Figure 3A).

Comparison of FhaA with the products of the *papC* gene family (Figure 3B) revealed 26.5% amino acid identity between PapC and FhaA, and 6% conservation among all known members of the PapC family. The sequence similarities are unevenly distributed and mainly clustered at the N-terminal end of the proteins. Four cysteine residues found in all the members of the family are strictly conserved.

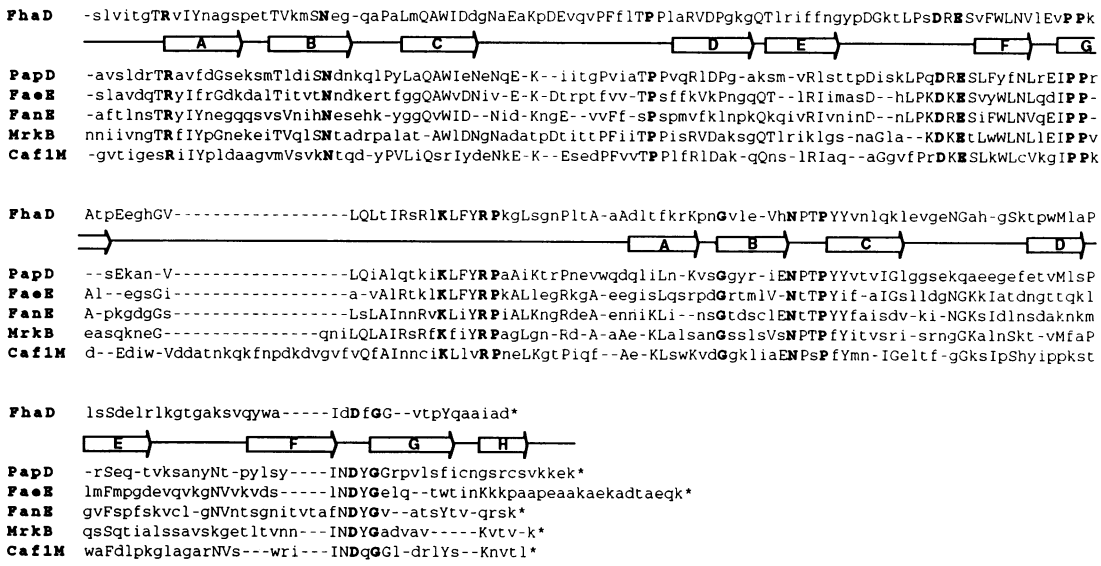
FhaE was found to be similar to members of the pilin protein family, including the *B.pertussis* fimbrial subunits Fim2, Fim3 and FimX (Figure 3C), with 7.5% strictly conserved residues among the proteins shown in Figure 3C, including two cysteines and the penultimate tyrosine generally conserved among all the known fimbrial subunits of the type 1 pilus family.

Requirement of the fhaB downstream genes for the biosynthesis of both FHA and the B.pertussis fimbriae

Since the Tn*5* insertion of the FHA$^-$ *B.pertussis* BP353 was mapped to the site shown in Figure 4, this insertion interrupts the *fhaA* gene product in its N-terminal region. If FhaA plays a role during the production of the *B.pertussis* fimbriae analogous to PapC in P pilus biogenesis, *B.pertussis* BP353 would be expected also to be deficient in the production of fimbriae. As shown in Figure 2C, no fimbrial subunit could be detected in *B.pertussis* BP353, using anti-fimbriae monoclonal antibodies, whereas anti-fimbriae reactive material with an electrophoretic mobility expected for the *B.pertussis* fimbrial subunits was clearly identified in the *B.pertussis* Tohama I control strain.

These results strongly suggest that the biosynthesis of fimbriae and FHA depend on common accessory genes. To rule out the possibility that FHA is directly required for the production of fimbriae, two *B.pertussis* FHA$^-$ mutants

A


FhaD -slvitgTRvIYnagspetTVkmSNeg-qaPaLmQAWIDdgNaEaKpDEvqvPFflTPPlaRVDPgkgQTlriffngypDGktLPsDRESvFWLNVlEvPPk
A
B
C
D
E
F
G
PapD -avsldrTRavfdGseksmTldiSNdnkqlPyLaQAWIeNeNqE-K--iitgPviaTPPvqRlDPg-aksm-vRlsttpDiskLPqDRESLFyfNLrEIPPr
FaeE -slavdqTRyIfrGdkdalTitvtNndkertfggQAWvDNiv-E-K-Dtrptfvv-TPsffkVkPngqQT--lRIimasD--hLPKDKESvyWLNLqdIPP-
FanE -aftlnsTRyIYnegqqsvsVnihNesehk-yggQvWID--Nid-KngE--vvFf-sPspmvfklnpkQkqivRIvninD--nLPKDRESiFWLNVqEIPP-
MrkB nniivngTRfIYpGnekeiTVqlSNtadrpalat-AWlDNgNadatpDtittPFiiTPPisRVDaksgQTlriklgs-naGla--KDKEtLwWLNLlEIPPv
Caf1M -gvtigesRiIYpldaagvmVsvkNtqd-yPVLiQsrIydeNkE-K--EsedPFvvTPPlfRlDak-qQns-lRIaq--aGgvfPrDKESLkWLcVkgIPPk

FhaD AtpEeghGV----------LQLtIRsRlKLFYRPkgLsgnPltA-aAdltfkrKpnGvle-VhNPTPYYvnlqklevgeNGah-gSktpwMlaP
A
B
C
D
PapD --sEkan-V----------LQiAlqtkiKLFYRPaAiKtrPnevwqdqliLn-KvsGgyr-iENPTPYYvtvIGlggsekqaeegefetvMlsP
FaeE Al--egsGi----------a-vAlRtklKLFYRPkALlegRkgA-eegisLqsrpdGrtmlV-NtTPYif-aIGslldgNGKkIatdngttqkl
FanE A-pkgdgGs----------LsLAInnRvKLiYRPiALKngRdeA-enniKLi--nsGtdsclENtTPYYfaisdv-ki-NGKsIdlnsdaknkm
MrkB easqkneG----------gniLQLAIRsRfKfiYRPagLgn-Rd-A-aAe-KLalsanGsslsVsNPTPfYitvsri-srngGKalnSkt-vMfaP
Caf1M d--Ediw-Vddatnkqkfnpdkdvgvfv QfAInnciKLlvRPneLKgtPiqf--Ae-KLswKvdGgkliaENPsPfYmn-IGeltf-gGksIpShyippkst

FhaD lsSdelrlkgtgaksvqywa-----IdDfGG--vtpYqaaiad*
E
F
G
H
PapD -rSeq-tvksanyNt-pylsy----INDYGGrpvlsficngsrcsvkkek*
FaeE lmFmpgdevqvkgNVvkvds-----lNDYGelq--twtinKkkpaapeaakaekadtaeqk*
FanE gvFspfskvcl-gNVntsgnitvtafNDYGv--atsYtv-qrsk*
MrkB qsSqtialssavskgetltvnn---INDYGadvav-----Kvtv-k*
Caf1M waFdlpkglagarNVs---wri---INDqGGl-drlYs--Knvtl*

B


FhaA pdmqaavnFdsaml-wGgangaDlSRFnySnalrPGnYiVDiyaNnypliRq--QvrfVaaQtSgq-glKtapavaCfTygqLeaMqVrlraldp-alvadl-kssGrCeVLg
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Cobtra DL--vVTV-EdasGhk-t----TtvyPvttlptLLRPGeveYnvavGR-Kssnyklkkpfadgen Gmfwm Gsvgygfdsttt LnaAsilhgkY-QAGgvsvtqaLGgfGAVSAgm
FhaA TlSRs--qlst-tGn-anGsSlRfsYSKtlSgtNtaiRl-A-tlRYSSsG-fwn-fadav-Nagpvetng--RNgrfglyslLgrerprgdfsvtlSq-pLggyGsl--YvsA-
PapC TqSvA--riegertf--qGkSwRlsYSKrfdnadaditf-A-GYRfSern--YmtmeQ-YlNarYrndys-SR-EkemytvtLnknv--adwntsF-n--L-qSeniK--v-A-
FaeD ayatAKyrdgshsGnkvql-Sw----SKqlettNtgLRv---swsrqSee--Yegms-sf-dptelwsq--S-Nhgrrtkdewnagisqpvggl-FS---LsvSGWqrsYypAs
FanD niSRAKnqletdqGystsan-----YSKnv-gaNgnLqii--GYkfSSeG--Y-t--Q-YaNfdYraprkdkk-EkeryevtLtqqf--pasnv-F----LsvtGWkK-fy---
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FhaA --lrrtYWgssrvdqqtqlgySTQv-grVgVnLdvSrtenrrst--ehqvmLNlSIPLy--G-atssgvvtgSlartgSapvqq-svNysgmsgerDqytY-GLgvqraGtsa-
PapC --v--dahriivrfpyvpVcL-TavlqGVaVgLsaSrSk-ylgrdnD-SayLriSvPL---Gtgt--asYSgSms--ndryvnmaGytdtfndgldsyslnaGLnsgggltsqr
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Cobtra ------YWwmkgkaiGgdVSLSTtildGVsVfLngSySkRpyldkpDyStSLsfSIP-FtlGgiR---hYSstglsynSsgrm--GmNsGvSaSptDrlsYglntnlsdkgdrs
FhaA qyaLNgSwSgtyg--evSgqlthGR-SysqyqingSGG--lvahagGVtfgqyqagTiGliqaEaaagakvvNtrnaavDrsGYglVs-LtpYslNeVelspqdLPlDVqLesT
PapC q--iNayYShrsplaNlSaniaslqkgytsfGvsaSGGatitgkgaalhaggmsggTrllvdtd-GVgGvpvdgGqvvTnrwGtgVVtdissYyrNttsvDlkrLPDDVEaTrs
FaeD sSyLNaSYSgdraylNgvlnhSQsggSqwfclgqrfGTgssGgerhhVqphdrr--hgGggecE-GhaGsegdsGdgqTDSdGnlVVP-LnsYdwNtVtIDtgtLPlstELTnT
FanD vS-LstnYtssmfrt--SasvSknRnStnaSaqi--GGaIiGvkdgGVmltsmssnsvaivqmE-GlaGyaftnG-vesDwrGriAYP-mttYmdNdiqIstdkLPsniELTdn
MrkC va-LNgSYrtpwttlNgSy--SQGegyr-qSGvgaSGTlIahqhgvvfspetgptmalieakdaaGVmlpgsp-Gtri-DSnGYailPyLrpYriNsVeIDpkgsnDDVafgsT
Cobtra lSg-NlSYgfdaiqtNmml--SQGRdnttvSGs-vSGT-IlGtadsGlmmtketgnTlGvarip-GVkGvringsap-TnSkGYtVVn-LsdYslNrVsvDmenvPDDlELqtT
FhaA VeqViPraaAVValrFptrhdvaamLvaepgseGa-LvFGTeVrdgaGkvvG-VAGqGaSaLvrgVsASG--TLeVtraDGsi-CratydlksagqavhglprialaCapqg...
PapC VvesalTEgAigyrkFsvlkgkRlfailrl-aDGsqpPFGasVtsekGrelGmVAdeGlawLs-gVt-pGe-TLsVn-wDGkiQCq----Vnvpetaisd-qqlllpCtpqk*
FaeD sqkVVPTdkAVVwmpFdalkvkRylLqVkqr-DGefvPgGTwardskntplGFVAnNGv-LmiNtVDApGdiTL------G--QCri---paarlqd-t-EKlQEitCE*
FanD VetiVPTnrAikLqkikyknmsRhvLkVydk-nGfviPmGTaVknsnGeiisFVnnNGiSLL-N-iDknder---V--ffG--sCti--stsglkdnls-E-iQEvnCE*
MrkC VaqVVPwEgsVVKvsFdttlqnnitLrarq-anGlpLPFaatifgpsGkeiGvV-GqG-Smmfis-DASap-katV-kwsGg-QCs----Vels-----qEKtkEtlC-r*
Cobtra sfnVVPTEkAVVyreFgaehvlRyiLrVker-DGriLngGsaqteq-GldaGFiAGNGv-LLmNml--SapsrvsVergDGsv-Ch--fsVkgivpn-tg-KvQEvyCE*

Fig. 3. Alignments of FhaD with the products of the *papD* gene family (**A**), of FhaA with the products of the *papC* gene family (**B**) and of the FhaE C-terminal domain with fimbrial subunits (**C**). Amino acids conserved among at least 50% of the aligned sequences are shown by upper-case letters and residues conserved among all aligned sequences are in bold. Hyphens represent gaps to allow for optimal sequence alignments. Asterisks indicate the C-terminal ends of the proteins. The arrow boxes in A represent the β strands of PapD (Holmgren and Branden, 1989). Letters in the arrow boxes give the order of the β strands in each of the two PapD domains. The amino acid sequences compared with FhaD are the chaperone proteins PapD of the *E.coli* P pilus (Lindberg *et al.*, 1989), FaeE of the *E.coli* K88 pilus (Bakker *et al.*, 1991), FanE of the *E.coli* K99 pilus (Bakker *et al.*, 1991), MrkB of the type 3 pilus of *K.pneumoniae* (Allen *et al.*, 1991) and Caf1M of the *Yersinia pestis* envelope antigen F1 (Galyov *et al.*, 1991). The amino acid sequences compared with FhaA were the outer membrane proteins PapC of the *E.coli* P pilus (Norgren *et al.*, 1987), FaeD of the *E.coli* K88 pilus (Mooi *et al.*, 1986), FanD of the *E.coli* K99 pilus (Roosendaal and de Graaf, 1989), MrkC of the type 3 pilus of *K.pneumoniae* (Allen *et al.*, 1991), the *Salmonella typhimurium* cobalamin transport protein (Cobtra, Rioux *et al.*, 1990). The amino acid sequences compared with FhaE are the *B.pertussis* fimbrial subunits Fim2 (Livey *et al.*, 1987), Fim3 (Cuzzoni *et al.*, 1990) and FimX (Pedroni *et al.*, 1988), the major *E.coli* F17 fimbrial subunit F17-A (Lintermans *et al.*, 1988) and the adhesin MrkD of of the type 3 pilus of *K.pneumoniae* (Allen *et al.*, 1991).

(SK16 and SK75), generated by Tn*PhoA* transposition (Knapp and Mekalanos, 1988), were analyzed for the production of fimbriae and FHA. The results indicated that both strains produce fimbriae, but no FHA (data not shown). Therefore, the absence of fimbriae in *B.pertussis* BP353 or in BPMC is not due to the absence of FHA in these strains, but caused by the mutations in the FHA accessory genes. The formal possibility that the lack of fimbriae formation *per se* interferes with the FHA biogenesis is unlikely, since it has previously been shown that insertional inactivation of the structural fimbrial subunit genes does not affect FHA production (Mooi *et al.*, 1988).

The fha gene structure

Since *fhaB* and the FHA/fimbriae accessory genes are clustered, it was of interest to investigate whether they could be expressed as part of the same polycistronic operon. The expression of the accessory genes can easily be assessed by monitoring the presence of fimbrial subunits. Since *B.pertussis* BPMC contains intact *fhaD*, *fhaA* and *fhaE* cistrons, but produces neither FHA nor fimbriae (as summarized in Figure 4), its *fhaB* deletion may have removed the promoter(s) required for *fhaDAE* expression, and/or intergenic sequences between *fhaB* and *fhaD* that would be essential for FHA and fimbriae production.

To distinguish between these possibilities, two additional *B.pertussis* mutant strains were constructed with either a deletion of the 1620 bp *Eco*RI–*Sph*I fragment containing the 3′ end of *fhaB* and the entire *fhaB–fhaD* intercistronic region, or a deletion of the 10 kb *Eco*RI fragment spanning ~90% of *fhaB* (Figure 4). These strains were called *B.pertussis* BPGR1 and *B.pertussis* BPGR4, respectively.

As depicted in Figure 4, BPGR1 produces both FHA and fimbriae, albeit significantly less than the Tohama I strain. The FHA secreted by BPGR1 shows a molecular mass similar to that secreted by the Tohama I strain. Analysis of the cell-associated material showed that BPGR1 cells contain more low molecular weight FHA polypeptides than the Tohama I cells, indicating that the BPGR1-produced cell-associated FHA is less stable than the one produced by the Tohama I strain. The level of fimbriae production by *B.pertussis* BPGR4 is similar to that observed for the Tohama I strain, and significantly higher than for the BPGR1 strain. As expected, BPGR4 does not produce any detectable FHA.

These results show that the *fhaB–fhaD* intercistronic region is not absolutely required for the production of FHA or fimbriae. In addition, expression of the *fhaB* downstream cistrons can be driven by the promoter that also controls the transcription of *fhaB*. Therefore they are most likely part of the same polycistronic operon. However, the expression levels of the accessory genes are higher when the intercistronic region is conserved, as evidenced by the increased production of fimbriae, suggesting that a second promoter is located upstream of *fhaD*.

The presence of a promoter between *fhaB* and *fhaD* was confirmed by primer extension analysis of total RNA extracted from *B.pertussis* Tohama I using an oligonucleotide complementary to the 5′ end of the *fhaD* coding strand. As shown in Figure 5, transcription of the accessory genes starts at a T residue, located 565 nucleotides upstream of the ATG start codon of *fhaD*.

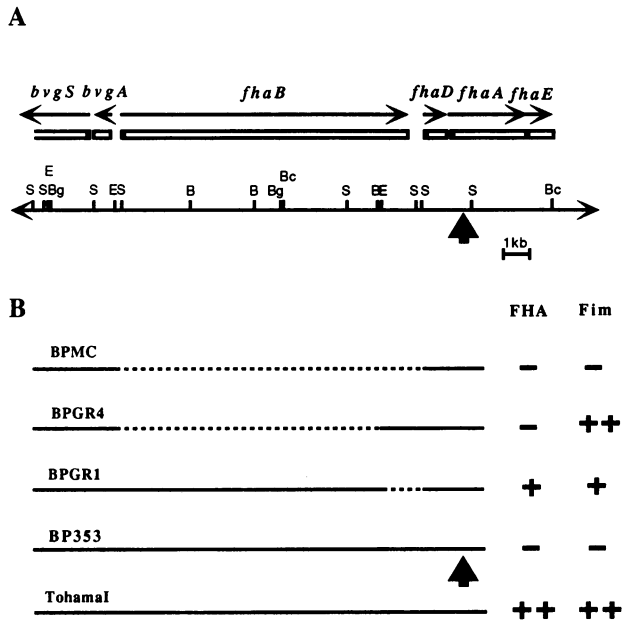

Fig. 4. Gene structure of the *fha* locus. (**A**) Open boxes and arrows show the lengths and directions of the *fha* or *bvg* ORFs above a partial restriction map of the locus. The restriction sites shown are the same as in Figure 1, with the addition of Bc, *BclI*. The large vertical arrow indicates the position of the Tn*5* insertion in *B.pertussis* BP353. (**B**) Deletion analysis of the *fha* locus showing the FHA and fimbriae (Fim) phenotypes. ++ indicates high levels, + indicates low but detectable levels, and − indicates undetectable levels of the respective proteins found in the different *B.pertussis* strains (*B.pertussis* BP353 contains very small amounts of cell-associated FHA, only detectable with polyclonal antibodies). The dotted lines represent the DNA portions that are deleted in the various strains, with reference to the restriction map shown in (A).

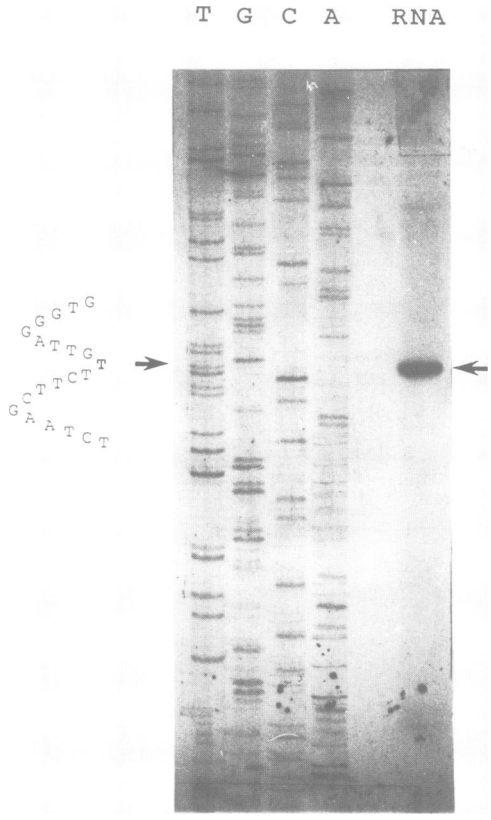

Fig. 5. Primer extension analysis of the *B.pertussis* mRNA. Total *B.pertussis* Tohama I RNA (10 μg) was annealed with the 5′ end-labeled oligonucleotide AATGAATAGCGGAACCATCCAGA-TTGGCTT, and transcribed by 200 U Moloney murine leukemia virus reverse transcriptase. In parallel, DNA sequencing reactions were performed on the M13mp18 subcloned DNA (non-coding strand) using the same but unlabeled oligonucleotide and [α-^{35}S]dATP. The products of the reverse transcription (lane RNA) and the DNA sequencing (lanes T, G, C and A) reactions were subjected to electrophoresis on a 6% polyacrylamide−urea gel and autoradiography. The transcriptional start site is indicated by the arrows.

Discussion

The translocation of large proteins through two membranes into the extracellular space, either as free molecules or as molecules attached to the outer surface of the organism represents one of the major challenges for Gram-negative bacteria. For this purpose these microorganisms have generally developed complex secretion strategies (for review see Hirst and Welch, 1988). *B.pertussis* is an attractive model system to study extracellular secretion mechanisms of large complex proteins, because several such proteins have been shown to be secreted by this organism into the culture medium. These proteins include PTX, adenylate cyclase/hemolysin, dermonecrotic toxin and FHA. The last protein can be found secreted into the culture medium, as well as attached to the outer surface of the bacterium. With respect to the latter characteristic, they somewhat resemble the Gram-negative pili or fimbriae. In the early literature, FHA was sometimes referred to as fimbrial hemagglutinin (Arai and Munoz, 1979).

Efficient production and secretion of FHA from *B.pertussis* require the expression of accessory genes, located downstream of *fhaB*. Three of them, named *fhaD*, *fhaA* and *fhaE*, have been identified in this study. Mutational inactivation of these genes by either transposon insertion or site-directed deletions results in an FHA negative phenotype. In addition, the same mutations also affect the production of the *B.pertussis* fimbriae, suggesting that this organism possesses common accessory genes responsible for the proper biogenesis of two different filamentous structures lacking significant sequence similarity.

The *fhaD* and *fhaA* gene products are found to share striking sequence similarities with the products of the *papD* and *papC* gene families, respectively. The *papD* and *papC* genes are essential for the biogenesis of the *E.coli* P pili (see Hultgren *et al.*, 1991), since mutations in these genes abolish piliation. *PapD* was shown to code for a chaperone protein, and *papC* for an outer membrane translocation protein. The sequence similarities of FhaD and FhaA with PapD and PapC strongly suggest functional similarities, which is supported by the fact that mutations in the *fha* locus abolish the *Bordetella* fimbriae production in addition to the production of FHA.

The PapD chaperone protein interacts with the subunits of the Pap pilus by forming heterodimers and transports these subunits through the periplasmic space in an assembly-competent form (Kuehn *et al.*, 1991). The crystal structure of PapD has been resolved at 2.5 Å resolution by Holmgren and Branden (1989). The protein was found to adopt an immunoglobulin fold that consists of two globular β barrels composed of antiparallel β pleated sheets. Like other members of the PapD family, the structure of FhaD can be

```
          VAtVEVVPRPKVETAQPLPPRPVaA
          VAKVEVVPRPKVETAQPLPPRPVvA
          VAKVtkaPpPvVETAQPLPPvkpqk

Consensus VAKVEVVPRPKVETAQPLPPRPV-A
```

Fig. 6. Tandem repeats at the C-terminal end of FhaB. Amino acid residues conserved among at least two of the repeats are shown by upper-case letters. Bold letters show amino acid residues conserved among all three repeats. The sequences were taken from Domenighini *et al.* (1990).

predicted using the amino acid sequence similarities to PapD and the known structure of this protein (Figure 3A). The degree of amino acid sequence similarity between FhaD and PapD is particularly important in the hydrophobic core of the molecules and in those areas that are thought to maintain the overall immunoglobulin-like fold. A high degree of sequence similarity is also found in the loop region that connects the two β barrels. This region also contains the highest concentration of amino acids conserved among all members of the family analyzed. On the other hand, little sequence similarity is found in those loops that are thought to be involved in specific binding to the pilin proteins, as would be expected, since the *E.coli* P pilin proteins are different from the *B.pertussis* fimbrial subunits or FHA. These observations are in agreement with the hypothesis that FhaD represents the *B.pertussis* periplasmic chaperone protein. Unlike for the *E.coli* pili, however, mutations in the accessory gene cluster that would still allow the synthesis of the chaperone protein do not appear to result in an accumulation of cell-associated fimbrial subunits in *B.pertussis* detectable by monoclonal antibody BPA10. This may be due to the lack of reactivity or sensitivity of the antibody with a precursor form of the fimbrial subunit, or to a rapid degradation of the periplasmic proteins in *B.pertussis* in spite of the presence of FhaD. Proteins trapped in the periplasm of *B.pertussis* are known to be rapidly degraded (Antoine and Locht, 1990; Pizza *et al.*, 1990), suggesting a high level of periplasmic protease activity in this organism. FhaD may not be sufficiently stabilizing to allow for high levels of periplasmic fimbrial subunit accumulation. Alternatively, other genes may also be involved in fimbriae production that might be affected by a polar effect of the described mutations. Current investigation in our laboratory aims at the identification of the genes located downstream of *fhaE*.

In comparison with PapD, much less is known about the structure of PapC or other members of this family. Therefore, amino acid alignments of these proteins are of limited use for the study of the structure of FhaA. No significant hydrophobic stretches could be identified in FhaA that would be long enough to span a lipid bilayer. The absence of long hydrophobic stretches is common for outer membrane proteins (Nikaido and Vaara, 1985) and is also found for the other members of the PapC family. Since biogenesis of both fimbriae and FHA was abolished by mutations that inactivate FhaA, we presume that this protein plays a role analogous to that played by PapC as a specific pore protein that facilitates translocation through the outer membrane of *B.pertussis*.

The *fhaA* cistron is followed by *fhaE*, whose predicted protein sequence contains sequence similarities with pilin proteins, including the *B.pertussis* fimbrial subunits. However, the FhaE protein is significantly longer than the *B.pertussis* fimbrial subunits with an extension of the N-terminus. Interestingly, the amino acid similarity between FhaE and the *Bordetella* fimbrial subunits is highest at the fimbrial N-terminal and C-terminal ends. In addition, the two cysteine residues and the penultimate tyrosine, conserved in almost all the known pilin subunits, are also found in FhaE. The N-terminal and C-terminal regions of the pilin subunits are thought to be involved in their interaction with the chaperone proteins. It is therefore tempting to speculate that the pilin-like C-terminal extension of FhaE may serve to interact with FhaD. The interactions between PapD and the P pilin subunits have been suggested to be of ionic nature, mutually neutralizing the charges of the basic PapD and the acidic pilin proteins (Kuehn *et al.*, 1991). Consistent with this hypothesis, the calculated pI value of FhaE is slightly basic (pI 7.13), whereas that of FhaD is slightly acidic (pI 6.63). The N-terminal extension of FhaE as compared with the *B.pertussis* fimbrial subunits, as well as its sequence similarities to some adhesins, such as the *Klebsiella pneumoniae* MrkD protein (Gerlach *et al.*, 1989), suggests that *fhaE* may code for a putative fimbrial adhesin. In comparison with the *K.pneumoniae mrk* gene cluster, the gene order in the *fha* locus would be consistent with this hypothesis. However, whether *fhaE* indeed codes for a minor fimbrial adhesin subunit and its role in the biogenesis of FHA and the fimbriae await further investigation.

Deletion analysis of the *fha* locus suggests that the FHA accessory genes can be expressed under the control of the *fhaB* promoter in a polycistronic operon, as well as under the control of their own upstream promoter. Indeed, primer extension analysis of *B.pertussis* RNA revealed the presence of a promoter between *fhaB* and *fhaD*, 565 nucleotides upstream of the ATG start codon for *fhaD*. The role of such a long 5′ non-translated region is not known at present; however, this region was found to be relatively AT-rich containing several T strings. T-rich portions can also be found in the *bvg* promoter, known to be regulated by transcription factors (Scarlato *et al.*, 1990; Roy and Falkow, 1991). Whether the expression of *fhaDAE* is regulated at the transcriptional or translational level remains to be investigated. The presence of multiple promoters in the *fha* locus is reminiscent of the gene structure of the *pap* locus.

Although *B.pertussis* BPGR1 produces lower amounts and less stable cell-associated FHA than the Tohama I strain, no qualitative difference was detected between the FHA secreted from Tohama I and that secreted from BPGR1. This observation indicates that the C-terminal truncation does not completely abolish secretion and proper cleavage of the 370 kDa precursor to generate the 220 kDa mature protein. The quantitative differences can be accounted for by the poor expression of the *fhaB* downstream genes in BPGR1 as compared with the Tohama I strain. The lack of stability of the cell-associated FHA however, could be the result of the C-terminal truncation of the *fhaB* ORF. Domenighini *et al.* (1990) have speculated that the $(PK)_5$ sequence found in this region may be involved in the anchoring of the FHA molecule in the periplasm, similar to what is observed with the streptococcal M protein. This is consistent with our data, since deletion of this sequence would be expected to alter the periplasmic stability of the protein, without necessarily abolishing the proper secretion and cleavage. In addition to the PK motif, three tandem repeats each containing 25 amino acid residues (Figure 6) were also found. Like the PK motif, these repeats are rich in proline residues (24%). Proline-rich repeats with some sequence similarities to the FHA

protein (five out of eight amino acid residues identical) can also be found in other adhesive cell surface structures, such as the streptococcal cell surface antigen I/II (Kelly *et al.*, 1989). More precise deletion experiments are needed to discriminate between the influence of the PK motif and that of the proline-rich repeats in the stability of cell-associated FHA. The C-terminal domain of FHA thus contains structures responsible for cell-associated FHA stability, whereas the N-terminal domain of FHA contains the major antigenic region, as well as receptor binding sites.

Materials and methods

Bacterial strains, plasmids and growth media

The streptomycin and nalidixic acid resistant *B.pertussis* Tohama I (*Sm*^{r} *Nal*^{r}) strain has been described previously (Antoine and Locht, 1990). *B.pertussis* BP353 (Weiss *et al.*, 1983) was provided by S.Falkow (Stanford University, Stanford, CA, USA). FHA$^-$ Tn*PhoA* mutants *B.pertussis* SK16 and SK75 (Knapp and Mekalanos, 1988) were provided by J.J.Mekalanos (Harvard Medical School, Boston, MA, USA). *E.coli* TG1 was purchased from Amersham Corp. (Aylesbury, UK). *E.coli* S17-1 (Simon *et al.*, 1983) was provided by A.Mercenier (Transgène, Strasbourg, France).

Plasmids pRIT13075, pRIT12990 and pRIT12988 were described previously (Delisse-Gathoye *et al.*, 1990). pUC18 and pUC19 as well as M13mp18 and M13mp19 were purchased from Pharmacia, Inc. (Piscataway, NJ, USA). pSS1129, a gentamicin resistant derivative of pRTP1 (Stibitz *et al.*, 1986) was provided by S.Stibitz (Food and Drug Administration, Bethesda, MD, USA). *E.coli* was grown in LB broth or minimal medium (Maniatis *et al.*, 1982). Antibiotic resistant *E.coli* were selected with 100 μg/ml of ampicillin. *B.pertussis* strains were grown on Bordet Gengou (BG) agar (Bordet and Gengou, 1906), or in modified Stainer Scholte (SS) medium containing 1 g/l of 2,6-*O*-dimethyl-β-cyclodextrin (Imaizumi *et al.*, 1983) and the relevant antibiotics at the following concentrations: streptomycin, 100 μg/ml; gentamicin, 10 μg/ml; and nalidixic acid, 50 μg/ml. BG agar contained 36 g/l BG agar (Difco Laboratories, Detroit, MI, USA), 1% glycerol and 20% defibrinated sheep blood.

Construction of mutant B.pertussis strains

Bordetella pertussis BPMC was constructed by two successive events of homologous recombination using pMC2. To generate pMC2, the 1.6 kb *Sph*I DNA fragment downstream of *fhaB* was first isolated from pRIT12990 (Delisse-Gathoye *et al.*, 1990) and subcloned into the *Sph*I site of pUC18. The 1.6 kb *Eco*RI–*Hin*dIII fragment of the resulting recombinant plasmid was then inserted into pSS1129, previously digested with *Eco*RI and *Hin*dIII to yield pMC1. The 2.5 kb *Eco*RI fragment containing the beginning of the *bvg* gene upstream of *fhaB* was then isolated from pRIT13075 and inserted into the *Eco*RI site of pMC1 to yield pMC2. This plasmid was introduced into *B.pertussis* Tohama I (*Sm*^{r} *Nal*^{r}) and clones with pMC2 integrated into their chromosome were selected on BG plates containing nalidixic acid and gentamicin. The gentamicin resistant clones were then plated on BG agar containing streptomycin to select for a second homologous recombination event. After the selection steps 100 hemolytic *B.pertussis* clones were analyzed by colony hybridization using the 4.5 kb *Eco*RI–*Bam*HI fragment containing the pertussis toxin gene (Locht and Keith, 1986) or with a 2.3 kb *Bam*HI fragment isolated from pRIT13075 and containing a central part of *fhaB*. One clone that hybridized with the *ptx* gene probe but not with the *fhaB* gene probe was further analyzed for the proper deletion of *fhaB* by Southern blot using both the 5′ and 3′ flanking regions and taking advantage of the newly introduced pUC18-derived restriction sites. This strain was named *B.pertussis* BPMC.

Bordetella pertussis BPGR1 was constructed by two successive homologous recombination events as described above, using pGR3, a pSS1129 derivative, and *B.pertussis* BPMC as the parent strain. pGR3 was generated by first isolating the 4.2 kb *Bgl*II–*Hin*dIII fragment from pMC2, and inserting it into pGR1, previously digested with *Bam*HI and *Hin*dIII, thereby yielding pGR2, and then inserting the 10 kb *Eco*R1 fragment containing the major portion of *fhaB*. pGR1 was generated by digesting pSS1129 with *Eco*RI, filling in the ends and religating the plasmid.

To construct *B.pertussis* BPGR4, the pSS1129 derivative pGR5 was used. This plasmid was generated by exchanging the 1730 bp *Eco*RI–*Hin*dIII fragment from pGR2 with the 2170 bp *Eco*RI–*Hin*dIII fragment from pGR4, a plasmid produced by inserting the 2170 bp *Eco*RI–*Pst*I fragment from pRIT12988 into pUC18 previously digested by *Eco*RI and *Pst*I. *B.pertussis* Tohama I *Sm*^{r} *Nal*^{r} was used as the parent strain to generate *B.pertussis* BPGR4 by homologous recombination as described above.

The DNA from *B.pertussis* BPGR1 and BPGR4 was analyzed by Southern blot to ensure that the correct deletions had occurred. Hemolysin tests and Western blot analysis with anti-PTX antibodies indicated that in these strains the activity of the *bvg* genes was not affected.

The pSS1129 derivatives were introduced into *B.pertussis* either by electroporation as described elsewhere (R.Antoine and C.Locht, in preparation), or by conjugation as described earlier (Antoine and Locht, 1990) using *E.coli* S17-1 as a donor strain.

DNA methodology

Restriction enzymes, T4 DNA ligase, T4 DNA polymerase and other DNA modifying enzymes were purchased from Pharmacia-LKB (Uppsala, Sweden), Boehringer (Mannheim, Germany), New England Biolabs (Beverly, MA, USA), Bethesda Research Laboratories (Gaithersburg, MD, USA) or Amersham Corp., and used as recommended by the suppliers. All other DNA manipulations were performed under standard conditions, as described by Maniatis *et al.* (1982). Southern blot analysis and colony blot hybridization were performed with non-radioactive DNA probes labeled with digoxigenin-dUTP and the development was done according to the instructions of the supplier (Boehringer, kit No. 1093 657). Total DNA was isolated from the *B.pertussis* strains by the method described by Hull *et al.* (1981).

DNA sequencing

The nucleotide sequence was determined by sequencing both strands using the dideoxy chain termination method. The DNA fragments were subcloned into M13mp18 or M13mp19 and then introduced into *E.coli* TG1. Purified single-stranded DNA was then sequenced using the fluorescent universal primer. Alternatively, the sequence of other regions was determined using the double-stranded plasmid DNA and various synthetic oligonucleotide primers hybridizing to internal regions of the plasmid. The sequencing was performed using the Automated DNA sequencer (Applied Biosystem, Foster City, CA, USA) and either the Taq dye primer sequencing kit (Applied Biosystem, reference number 401119) or the Taq Dye Deoxy™ Terminator sequencing kit (Applied Biosystem, reference number 401150), following the instructions of the supplier. Alternatively, the DNA was sequenced using [α-^{35}S]dATP (800–1500 Ci/mmol, New England Nuclear) and the DNA T7 Sequencing kit (Pharmacia-LKB), following the instructions of the supplier. The DNA and protein sequence were analyzed using either DNA Strider (Marck, 1988) or DNASTAR (DNASTAR, Inc., UK). The CODONPREFERENCE program was used with the recently published codon usage of *B.pertussis* (Wada *et al.*, 1991).

RNA techniques

RNA was extracted from *B.pertussis* using the method described by Laoide and Ullmann (1990). For the primer extension analysis the 30mer synthetic oligonucleotide whose sequence is shown in the legend of Figure 5, was first labeled using [γ-^{32}P]ATP (5000 Ci/mmol; Amersham) in a total vol of 20 μl containing 500 ng of oligonucleotide, 10 U polynucleotide kinase (Boehringer), 20 mCi [γ-^{32}P]ATP, 0.1 M Tris–HCl (pH 8.0), 10 mM $MgCl_2$ and 7 mM dithiothreitol (DTT). After incubation at 37°C for 45 min, 180 μl H_2O and 20 μg carrier tRNA (Boehringer) were added and the nucleic acids were ethanol-precipitated. The precipitated material was then resuspended in 50 μl bidistilled RNase-free H_2O.

Radiolabeled oligonucleotides (10^6 c.p.m.) were annealed with 10 μg of total *B.pertussis* RNA in a 20 μl vol (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT) by heating the mixture at 80°C for 3 min and allowing the temperature to drop slowly to 54°C. 10 U (1 μl) RNasin (Promega Corp. Madison, WI, USA) were added and the incubation was continued for 30 min. The mixture was then incubated at −20°C for 20 min. 10 μl of the annealing mixture were then incubated in a total volume of 25 μl containing 10 mM DTT, 40 μM dNTPs, 200 U Moloney murine leukemia virus reverse transcriptase (Gibco, BRL, Ghen, Belgium), 10 U RNasin, for 30 min at 45°C. The reaction was stopped by the addition of 190 μl H_2O, phenol/chloroform/isoamyl-alcohol extraction and ethanol precipitation. After precipitation the material was resuspended in 10 μl of loading buffer (90% formamide in TBE, Maniatis *et al.*, 1982) containing Bromophenol Blue and Xylene Cyanol as tracking dyes), denatured for 2 min at 90°C, and electrophoresed on a 6% polyacrylamide–8 M urea sequencing gel.

Analytical procedures

SDS–PAGE, using 12.5 or 10% slab gels, was carried out as described by Laemmli (1970). Western blot analyses using monoclonal antibodies were performed as described (Burnette, 1981). The monoclonal antibody P11B10 directed against the S2 subunit of PTX (Frank and Parker, 1984) was provided by Frank and Parker and the monoclonal antibody 12.1F9 (Delisse-

Gathoye *et al.*, 1990) against FHA came from I.Heron (Statens Seruminstitut, Copenhagen, Denmark), and anti-fimbriae monoclonal antibody BPA10, reactive with both Fim2 and Fim3 fimbriae, was provided by M.Brennan (Food and Drug Administration, Bethesda, MD, USA).

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Note added in proof

The sequences reported here are available from the EMBL/GenBank nucleotide sequence databases under accession number X66729. After this manuscript was submitted Willems *et al.* (*Mol. Microbiol.*, in press) identified the fimbrial accessory genes by analyzing mutants deficient in fimbrial production.