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Lack of Functional Complementation between *Bordetella pertussis* Filamentous Hemagglutinin and *Proteus mirabilis* HpmA Hemolysin Secretion Machineries

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The gram-negative bacterium *Bordetella pertussis* has adapted specific secretion machineries for each of its major secretory proteins. In particular, the highly efficient secretion of filamentous hemagglutinin (FHA) is mediated by the accessory protein FhaC. FhaC belongs to a family of outer membrane proteins which are involved in the secretion of large adhesins or in the activation and secretion of Ca^{2+}-independent hemolysins by several gram-negative bacteria. FHA shares with these hemolysins a 115-residue-long amino-proximal region essential for its secretion. To compare the secretory pathways of these hemolysins and FHA, we attempted functional transcomplementation between FhaC and the *Proteus mirabilis* hemolysin accessory protein HpmB. HpmB could not promote the secretion of FHA derivatives. Likewise, FhaC proved to be unable to mediate secretion and activation of HpmA, the cognate secretory partner of HpmB. In contrast, ShlB, the accessory protein of the closely related *Serratia marcescens* hemolysin, was able to activate and secrete HpmA. Two invariant asparagine residues lying in the region of homology shared by secretory proteins and shown to be essential for the secretion and activation of the hemolysins were replaced in FHA by site-directed mutagenesis. Replacements of these residues indicated that both are involved in, but only the first one is crucial to, FHA secretion. This slight discrepancy together with the lack of functional complementation demonstrates major differences between the hemolysins and FHA secretion machineries.

The whooping cough agent *Bordetella pertussis* secretes several virulence factors into the extracellular milieu. Among them, the 220-kDa filamentous hemagglutinin (FHA) is the major extracellular protein, secreted in a highly efficient manner (19). At least one accessory protein, named FhaC, is known to be required for FHA secretion (35). FhaC belongs to a growing family of similar accessory proteins involved in the secretion of virulence factors of various gram-negative organisms (Table 1). In addition to FhaC, this family comprises HpmB, ShlB, and HhdB, accessory proteins required for the secretion and the activation of their cognate Ca^{2+}-independent hemolysins HpmA, ShlA, and HhdA produced by *Proteus mirabilis*, *Serratia marcescens*, and *Haemophilus ducreyi*, respectively (24, 25, 32, 33). In addition, this family includes *Haemophilus influenzae* HMW1B and -2B, involved in the secretion of the HMW1A and -2A adhesins (3).

These approximately 60-kDa accessory proteins are predicted to be composed of a number of amphipathic β-strands, a feature shared with most outer membrane proteins (3–5). In agreement with the proposed outer membrane location of these proteins, FhaC was recently shown to be found at least in part in Sarkosyl-insoluble extracts (15).

Interestingly, the three closely related HpmA, ShlA, and HhdA hemolysins possess in their amino-proximal regions a 115-residue-long domain homologous to FHA (9). This highly conserved segment is hypothesized to interact with the cognate accessory protein. In agreement with this proposal, the deletion of this region totally abolishes the secretion of FHA (35), whereas several truncated forms of FHA which retain this region are secreted very efficiently in a FhaC-dependent manner (28). Small in-frame deletions as well as certain point mutations within this segment of ShlA affected both its secretion and activation (31). In particular, replacements of Asn-69 or Asn-109 in ShlA abolished its secretion and activation by ShlB, whereas alterations of other residues had much less drastic effects.

These two asparagine residues are among the strictly conserved residues of the three hemolysins and FHA, and they are contained within N(S/P)(N/H)L and NPNG(I/M) motifs (Table 1). The NPNGI sequence is also found in the HMWA adhesins. Except for the conservation of this short motif, the HMWAs are only distantly related to the other four proteins, even though an anti-FHA monoclonal antibody was found to cross-react with these adhesins (2).

In spite of the similarities shared by the secretion systems of the three hemolysins, FHA, and HMWA, they also show important differences. The hemolysins are most likely secreted with the help of classical signal peptides, whereas the N-terminal segments of FHA (15) and HMWA (3) do not function as typical signal peptides although they are proteolytically removed upon biogenesis. This N-terminal maturation appears to be much more extensive for HMWA than for FHA. In addition and unlike that of the hemolysins, the secretion of FHA involves extensive C-terminal processing of a large precursor (10, 27, 28). Finally, the hemolysins acquire hemolytic activity via activation by their cognate accessory proteins, whereas the adhesins are unlikely to require activation.

In view of these similarities and differences between the secretion systems of FHA and the hemolysins, we attempted cross-complementations between the FHA-FhaC and the

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TABLE 1. Family of secretory proteins

Organism	Secretory protein	Accessory protein(s)	Conserved motif(s)
Proteus mirabilis	Hemolysin HpmA	HpmB	ANSNL SNPNGI
Serratia marcescens	Hemolysin ShlA	ShlB	ANPNL ANPNGI
Haemophilus ducreyi	Hemolysin HhdA	HhdB	ANPHL VNPNGM
Haemophilus influenzae	Adhesin HMW1A	HMW1B; HMW1C	INPNGI
	Adhesin HMW2A	HMW2B; HMW2C	INPNGI
Bordetella pertussis	Adhesin FHA	FhaC	KNPNL ANPNGI

HpmA-HpmB secretion systems and report here the lack of functional complementation between them, although one of the conserved asparagines appeared to be essential for FHA secretion.

MATERIALS AND METHODS

Strains and plasmids. The relevant features of the *B. pertussis* and *Escherichia coli* strains and of the plasmids used in this study are shown in Table 2, and culture conditions were described previously (15).

Plasmids pBG4, pFJD6, pFJD8, pFJD9, pFJD11, and pFJD12 were described previously, as indicated in Table 2. pBG10 was constructed as follows. A 471-bp fragment of *hpmA* encoding the region of homology between HpmA and FHA was amplified by PCR with pWPM100 (34) as a template and the following oligonucleotides as primers: 5′ TACTGCAGGTTATTGGTGGTACGCAA GTC 3′ and 5′ TACTGCAGAATACGAGGAGCAATTAAGTC 3′, each containing a *Pst*I site (underlined). Following amplification, the PCR product was restricted by *Pst*I and cloned into the *Pst*I site of pUC18-4 (28), resulting in pUC18-4P. The 1.26-kb *Sal*I-*Bam*HI fragment isolated from pRIT13197 (9) was then inserted into the corresponding site of pUC18-4P to create pUC18-5P, and the 2.1-kb *Sph*I-*Bam*HI fragment from pUC18-5P was exchanged for its wild-type counterpart in pBG4 (28). To construct pEC11B, a 2.5-kb *Mlu*I-*Bam*HI fragment from pBG10 was cloned into the corresponding sites of pEC11 (15). pEC11B was then digested with *Xba*I, treated with Klenow enzyme, and then redigested with *Bam*HI. The resulting 2.8-kb fragment containing a translational fusion between the OmpA signal peptide coding sequence and the chimeric *hpmA-fha44* gene was cloned into pMMB91 (13), which had been digested with *Eco*RI, treated with Klenow enzyme, and redigested with *Bam*HI, so that the chimeric gene was brought under the control of the *tac* promoter. The resulting plasmid was called pFJD21. pFJD20 was obtained by digesting pWPM100 with *Eco*NI, treating it with Klenow enzyme, and redigesting it with *Xba*I. The resulting 3.9-kb *hpmA*-containing fragment was then cloned into the *Hin*cII and *Xba*I sites of pBBR1MCS (16), placing *hpmA* under the control of the *lac* promoter. pFJD23 was constructed by digesting pWPM100 with *Sau*96I, treating it with Klenow enzyme, and redigesting it with *Pst*I. The resulting *hpmB*-containing 1.85-kb fragment of pWPM100 was cloned into pMMB67HE, which had been digested with *Xba*I, treated with Klenow enzyme, and then redigested with *Pst*I, placing *hpmB* under the control of the *tac* promoter. pFJD30 was obtained by cloning the same fragment into pQE32, which had been digested with *Hin*dIII, treated it with Klenow enzyme, and redigested it with *Pst*I, placing *hpmB* under the control of the *tac* promoter. pFJD18 was obtained by digesting pFJD2 (15) with *Eco*RI, filling the site with Klenow enzyme, and redigesting with *Bcl*I. The resulting 2.2-kb *fhaC*-containing fragment from pFJD2 was cloned into pET22B (Novagen, Madison, Wis.), which had been digested with *Nde*I, treated with Klenow enzyme, and redigested with *Bam*HI, placing *fhaC* under the control of the T7 promoter. pFJD33 was constructed by cloning the 3.2-kb *Sal*I-*Xba*I fragment containing *shlB* from pMH1 into the corresponding sites of pET22B, placing *shlB* under the control of the T7 promoter.

Oligonucleotide-directed mutagenesis. The Sculptor in vitro mutagenesis system (Amersham RPN 1526) was used as recommended by the supplier to introduce the chosen mutations into the *fha44* gene. The template for site-directed mutagenesis was generated as follows. A 0.86-kb *Eco*RI-*Not*I fragment from pRIT13197 containing the promoter region and the 5′ end of *fhaB* was cloned into the corresponding sites of pBluescript KS$^+$, generating pCB1. This plasmid was then digested with *Acc*I and *Sac*I, and the insert was transferred into M13mp19 to give rise to ϕCB2. To create ϕEC1, the *Nci*I site present 29 bp 3′ of *Eco*RI in the 0.86-kb *Eco*RI-*Not*I fragment of ϕCB2 was eliminated with the oligonucleotide 5′-ACCCGCTCCC**T**GCCCGCC-3′ (The bold letter indicates the mismatch between the oligonucleotide and the template.) This construct was necessary to optimize the efficiency of further mutagenesis experiments.

In the recombinant phages ϕFha44-N137I and ϕFha44-N176I, codons 137 and 176 of the *fha44* gene were converted from AAC (asparagine) to ATC (isoleucine) by two mutagenesis reactions involving single-stranded ϕEC1 as the template and the mutant oligonucleotides 5′-GGTTGGGGATCTTGGTCAGC-3′ and 5′-CGTTGGGGATGGCGATGATG-3′, respectively (e.g., N137I represents the N-to-I mutation at position 137). The presence of the desired mutations and the absence of any other changes in the *fha44* portion of the recombinant phages were confirmed by DNA analysis with the T7 sequencing kit (Pharmacia Biotech).

The mutant *fha44* DNA segments were then introduced into the *fha44* expression vector pFJD9 as follows. The 0.86-kb *Eco*RI-*Not*I fragment of pFJD9 (15) was replaced by a 1.0-kb *Eco*RI-*Not*I fragment of foreign DNA, giving rise to pCB3. The 0.86-kb *Eco*RI-*Not*I segments of double-stranded ϕFha44-N137I and ϕFha44-N176I were then substituted in pCB3 for the 1.0-kb fragment. The subsequent digestion of plasmid DNA allowed a simple and reliable detection of the mutant Fha44-encoding vectors, which were named pFJD9-N137I and pFJD9-N176I, respectively.

To express the mutant *fha44* genes in *E. coli*, pFJD38 and pFJD39, which encode translational fusions of the OmpA signal peptide with Fha44-N137I and Fha44-N176I, respectively, were obtained as follows. The replicative forms of ϕFHA-N137I and ϕFHA-N176I were restricted with *Sph*I and *Not*I, and the resulting 0.5-kb *Sph*I-*Not*I fragments encompassing the mutated regions were purified and exchanged for their wild-type counterpart in pEC11 (15), yielding plasmids pFJD36 and pFJD37, respectively. After sequencing the inserts of these plasmids, pFJD36 and pFJD37 were digested with *Xba*I. The resulting protruding ends were filled in with Klenow enzyme and the plasmids were then redigested with *Bam*HI. The 2.7-kb fragments thus obtained were cloned into pMMB91 to place the translational fusions under the control of the *tac* promoter, resulting in pFJD38 and pFJD39.

Measurement of hemolytic activities. *E. coli* UT5600 transformed with pFJD20 alone or together with pFJD6 (*fhaC*$^+$), pFJD23 (*hpmB*$^+$), or pFJD30 (*hpmB*$^+$) or HMS174(DE3) transformed by pFJD20 (*hpmA*$^+$) alone or together with pFJD18 (*fhaC*$^+$) or pFJD33 (*shlB*$^+$) was grown in Luria-Bertani liquid medium with the required antibiotics to an absorbancy of 0.8 at 600 nm. For the UT5600 strains, the expression of the genes was induced by the addition of 1 mM (final concentration) IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h. For HMS174(DE3) strains, the expression of the T7 polymerase was induced by treatment with 2 mM (final concentration) IPTG for 1 h. After centrifugation of the cultures, the supernatant fractions were kept on ice, and the pellets were resuspended in the same volume of 0.9% NaCl as the initial culture and split into two parts. The first part was kept on ice, and the second part was washed in 0.9% NaCl and subjected to sonication on ice. The sonicates were clarified by a centrifugation at 7,000 × *g* for 15 min. Two hundred microliters of culture supernatants, washed whole cells, or clarified sonicates was mixed with 800 μl of a 1.25% suspension of extensively washed rabbit erythrocytes in 0.9% NaCl and incubated at 37°C for 30 min. The suspensions were then briefly centrifuged, and the absorbancy of the supernatants was determined at 540 nm. Two hundred microliters of culture medium, as a control for the culture supernatants, or 0.9% NaCl, as a control for whole cells or sonicate extracts, was mixed with the erythrocytes, and the mixture was processed as described above. The absorbancy at 540 nm of the controls was subtracted from that of the samples. The activities are expressed as the average absorbancy at 540 nm per 30 min of incubation for three to four separate experiments.

Protein analyses. To prepare the protein samples, liquid cultures of the various *E. coli* strains were grown in LB medium to an absorbancy of about 1 at 600 nm. The expression of the genes encoding the secretory and the accessory proteins was induced by the addition of IPTG at a final concentration of 1 mM for 3 h. Culture supernatants were then collected by centrifugation and concentrated by precipitation with 6% trichloroacetic acid and 20 mM deoxycholate where specified. Cell pellets were resuspended in 50 mM sodium phosphate buffer (pH 7) in one-fifth of the original volume of culture and sonicated on ice with 1-min pulses until the cell suspension became clear by visual inspection. The sonicates were centrifuged for 15 min at 7,000 × *g*. The amounts of total proteins present in the extracts were determined by measuring the absorbancy of these supernatants at 280 nm to correct for differences in sonication efficiencies.

Liquid cultures of *B. pertussis* containing the appropriate plasmids were grown in Stainer-Scholte medium supplemented with 0.1% dimethyl-β-cyclodextrin (Teijin Ltd., Tokyo, Japan) and the required antibiotics for 2 to 3 days until the late exponential phase. Cells and supernatants were separated by centrifugation. Where mentioned, cell pellets were sonicated as described above for *E. coli*. Culture supernatants of *B. pertussis* were not concentrated prior to analysis. For comparisons, the same volumes of supernatants from cultures having reached the same absorbancies at 600 nm were loaded onto 10 or 12% polyacrylamide gels

TABLE 2. Strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Relevant features	Reference or source
Strains		
B. pertussis		
BPSM	Sm^r Nal^r Tohama I derivative	21
BPGR4	BPSM derivative with a chromosomal deletion of *fhaB*	20
E. coli		
UT5600	Δ(*ompT-fepC*)	*E. coli* Genetic Stock Center (Yale University)
HMS174(DE3)	T7 polymerase under the control of a *lac UV5* promoter	Novagen, Madison, Wis.
Phages		
ϕCB2	900-bp *Acc*I-*Sac*I fragment from pCB1 containing the 5′ region of *fhaB* in M13mp19	This work
ϕEC1	ϕCB2 derivative with a mutation eliminating the *Nci*I site of the *fhaB* insert	This work
ϕFHA-N137I	N137I mutation in ϕEC1	This work
ϕFHA-N176I	N176I mutation in ϕEC1	This work
Plasmids		
Cloning vectors		
pBBR122	Broad-host-range cloning vector, Km^r	1
pBBR1MCS	Broad-host-range cloning vector derived from pBBR122, Cm^r	16
pBlueScript KS^+		Stratagene
pET22B	Promoter of phage T7 for high-level expression	Novagen
pMMB67	Broad-host-range vector containing the *tac* promoter, Amp^r	13
pMMB91	Broad-host-range vector containing the *tac* promoter, Km^r	13
pQE32	Expression vector containing the T5 promoter and *lac* operator sequences	Diagen
Intermediate constructs		
pRIT13197	6-kb *Eco*RI-*Bam*HI fragment containing the 5′ end of *fhaB* in pUC8	9
pUC18-3	1.26-kb *Sph*I-*Sal*I *fhaB* fragment in pUC18	28
pUC18-4	pUC18-3 with a deletion of internal 471-bp *Pst*I fragment	28
pUC18-4P	Similar to pUC18-3 but with 471-bp *Pst*I fragment replaced by the corresponding fragment of *hpmA*	This work
pUC18-5P	2.5-kb *Sph*I-*Bam*HI fragment of *fha44* with the *Pst*I fragment of *hpmA*	This work
pEC11	Translational fusion encoding OmpA signal peptide plus Fha44 (residues 3 to 862) in pACYC184	15
pEC11B	Similar to pEC11 except for the replacement of the Fha44 region of homology by that of HpmA	This work
pFJD2	Two tandem copies of *fhaC* on *Bcl*I fragments in pQE32	15
pWPM100	*hpmB* and *hpmA* in pUC19	34
pCB1	0.86-kb *Eco*RI-*Not*I fragment containing the 5′ end of *fhaB* in pBluescript KS^+	This work
pMH1	*shlB* in pACYC184	Gift of V. Braun
pFJD36	pEC11 derivative with the N137I point mutation	This work
pFJD37	pEC11 derivative with the N176I point mutation	This work
Expression plasmids		
pBG4	2.8-kb *Eco*RI-*Bam*HI fragment containing the 5′ end of *fhaB* (*fha44*) in pBBR122	28
pBG10	Hemolysin-homologous region of *fha44* replaced by the corresponding fragment of *hpmA*	This work
pFJD6	*fhaC* in pQE32	15
pFJD8	*fhaC* in pMMB67	15
pFJD9	*fha44* in pMMB91	15
pFJD9-N137I	pFJD9 derivative with the N137I point mutation	This work
pFJD9-N176I	pFJD9 derivative with the N176I point mutation	This work
pFJD11	Translational fusion encoding OmpA signal peptide plus Fha44 (residues 3 to 862) in pMMB91	15
pFJD12	Translational fusion encoding OmpA signal peptide plus Fha44 (residues 34 to 862) in pMMB91	15
pFJD20	*hpmA* from pWPM100 in pBBR1MCS	This work
pFJD21	Translational fusion encoding OmpA signal peptide plus the Fha44-HpmA chimera (residues 3 to 862) in pMMB91	This work
pFJD23	*hpmB* from pWPM100 in pMMB67HE	This work
pFJD30	*hpmB* from pWPM100 in pQE32	This work
pFJD33	*shlB* from pMH1 in pET22B	This work
pFJD38	Translational fusion encoding OmpA signal peptide plus Fha44 (residues 3 to 862) with the N137I point mutation in pMMB91	This work
pFJD39	Translational fusion encoding OmpA signal peptide plus Fha44 (residues 3 to 862) with the N176I point mutation in pMMB91	This work

and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Similarly, equivalent amounts of total proteins from sonicate extracts as determined by the absorbancy at 280 nm of the clarified protein solutions were subjected to SDS-PAGE. The proteins were electrotransferred onto nitrocellulose membranes and developed with either an anti-Fha44 polyclonal antiserum (15), an anti-FHA polyclonal antiserum (15), or an anti-HpmA polyclonal antiserum (kindly provided by R. Welch, Madison, Wis.). All antisera were used at a 500-fold dilution.

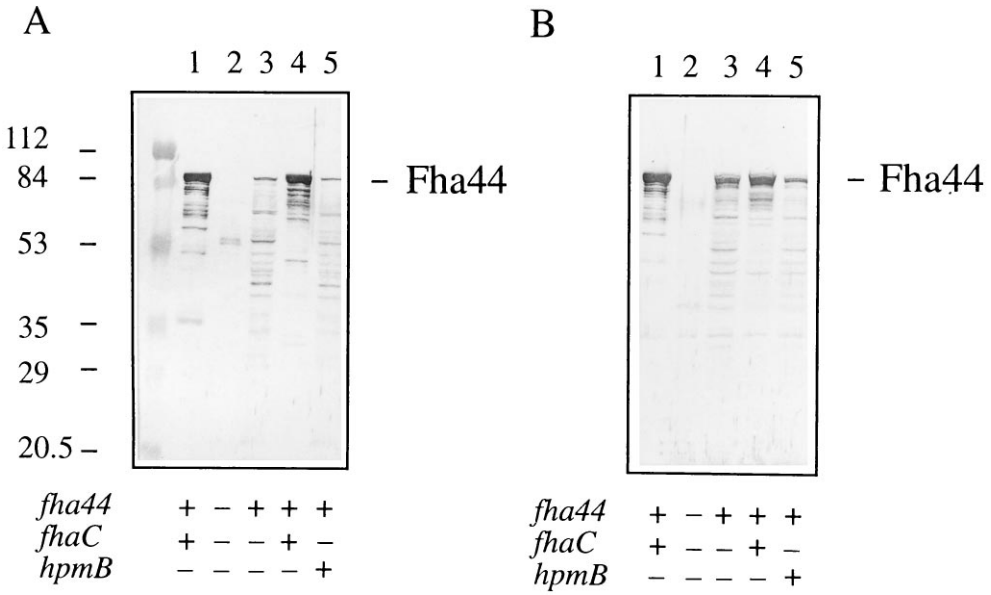

FIG. 1. Secretion of Fha44 in *E. coli*. Culture supernatants (A) and cell sonicates (B) of UT5600 (lanes 2), UT5600(pFJD12) (lanes 3), UT5600(pFJD12, pFJD6) (lanes 4), and UT5600(pFJD12, pFJD30) (lanes 5) were analyzed by immunoblotting with an anti-Fha44 polyclonal antiserum. The same-size volumes of 10-fold-concentrated supernatants were loaded in lanes 2 to 5 of panel A. In panel B, similar amounts of proteins from the clarified sonicates were loaded in lanes 2 to 5. Prestained marker proteins are shown in the leftmost lane of panel A, and their molecular sizes in kilodaltons are given in the left margin. Fha44 secreted in the culture supernatant of *B. pertussis* BPGR4(pBG4) is shown as a control in lanes 1.

Quantitation of secreted Fha44 was performed with an enzyme-linked immunosorbent assay as described previously (15).

RESULTS

Lack of complementation of *fhaC* by *hpmB*. Previous studies have indicated that the major secretion determinant of FHA is located in its amino-proximal region and that a truncated N-terminal FHA fragment, named Fha44, is secreted very efficiently in an FhaC-dependent manner by both *B. pertussis* and *E. coli* (15, 28). We therefore used Fha44 to examine whether the secretion machineries of FHA and the hemolysins are exchangeable.

In *E. coli*, Fha44 is efficiently secreted in an FhaC-dependent fashion only if the OmpA signal peptide is fused to its N-terminal end (15). The chimeric protein is encoded by pFJD12 (15). To determine whether HpmB can functionally replace FhaC for Fha44 secretion, *E. coli* UT5600(pFJD12) was transformed with pFJD30, containing *hpmB*. As shown in Fig. 1A, the presence of HpmB did not enhance the production of extracellular Fha44 over that of UT5600(pFJD12). Only very small amounts of Fha44 were detected in culture supernatants of *E. coli* UT5600(pFJD12) regardless of the presence of *hpmB* (Fig. 1A, lanes 3 and 5), in contrast to that detected in *E. coli* UT5600(pFJD12, pFJD6) containing *fhaC* in addition to *fha44* (Fig. 1A, lane 4). Anti-Fha44 immunoreactive proteins of the expected size were present in the cell sonicates of all strains that contained the Fha44-encoding gene (Fig. 1B, lanes 3 to 5). The low level of FhaC-independent secretion of Fha44 by *E. coli* most likely results from nonspecific leakage or slight cellular lysis, as observed previously (15). These observations suggest that HpmB cannot replace FhaC for efficient secretion of Fha44 in *E. coli*.

The absence of complementation was not caused by the lack of production or function of HpmB, since coexpression of *hpmA* and *hpmB* in UT5600(pFJD20, pFJD30) resulted in readily detectable extracellular hemolytic activity, whereas nonrecom-

TABLE 3. Hemolytic activities of various *E. coli* strains

Strain	HpmA	HpmB	FhaC	ShlB	Hemolytic activity[a]		
					Supernatants	Whole cells	Sonicates
UT5600	–	–	–	–	0	0	0
UT5600(pFJD20)	+	–	–	–	0	0	0
UT5600(pFJD20, pFJD6)	+	–	+	–	0	0	0.013 (0.002)
UT5600(pFJD20, pFJD23)	+	+	–	–	2.4 (0.65)	2.1 (0.65)	0.22 (0.12)
UT5600(pFJD20, pFJD30)	+	+	–	–	2 (0.4)	3.5 (0.5)	ND[b]
HMS174(DE3)	–	–	–	–	0	0.10 (0.04)	ND
HMS174(DE3, pFJD20)	+	–	–	–	0	0.013 (0.006)	ND
HMS174(DE3, pFJD20, pFJD18)	+	–	+	–	0	0	ND
HMS174(DE3, pFJD20, pFJD33)	+	–	–	+	1.72 (0.11)	1.84 (0.19)	ND

[a] Hemolytic activities are expressed as average absorbancies at 540 nm following a 30-min incubation of rabbit erythrocytes with supernatants, whole cells, or cell sonicates of the indicated strains. Standard deviations are given in parentheses.
[b] ND, not determined.

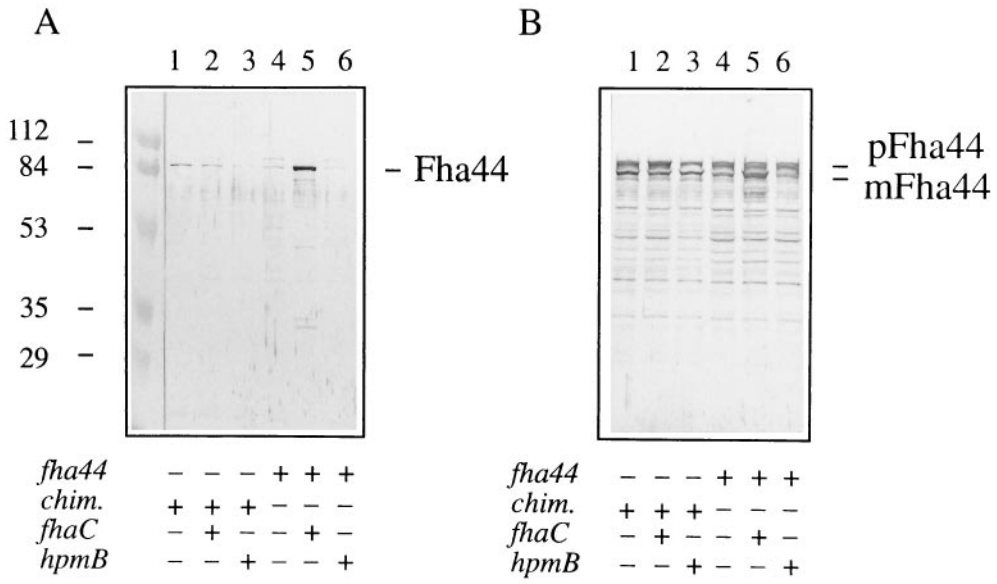

FIG. 2. Lack of secretion of a Fha44-HpmA chimera in *E. coli*. Culture supernatants (A) and cell extracts (B) of UT5600(pFJD21) (lanes 1), UT5600(pFJD21, pFJD6) (lanes 2), UT5600(pFJD21, pFJD30) (lanes 3), UT5600(pFJD11) (lanes 4), UT5600(pFJD11, pFJD6) (lanes 5), and UT5600(pFJD11, pFJD30) (lanes 6) were analyzed by immunoblotting with an anti-Fha44 polyclonal antiserum. The same-size volumes of 10-fold-concentrated supernatants were loaded in each lane of panel A. In panel B, similar amounts of proteins from the clarified sonicates were loaded. *chim*. denotes the *fha44-hpmA* chimeric gene. Note the presence of the mature (mFha44) and precursor (pFha44) forms of Fha44 derivatives in cell extracts, as already reported when pFJD11 was used (15). Prestained marker proteins are shown in the leftmost lane of panel A, and their molecular sizes in kilodaltons are given in the left margin.

binant UT5600 or UT5600(pFJD20) only expressing *hpmA* produced no detectable extracellular hemolytic activity (Table 3).

We attempted to determine whether HpmB could promote the secretion of FHA in *B. pertussis*. Since pFJD6 and pFJD30 do not replicate in *B. pertussis*, we cloned the *fhaC* and *hpmB* genes in pMMB67, under the control of the *tac* promoter, yielding pFJD8 and pFJD23, respectively. These plasmids were introduced into a *B. pertussis* strain with a chromosomal deletion of *fhaC*. Only FhaC, but not HpmB, was able to restore the production of extracellular FHA (data not shown). However, functional expression of *hpmB* from pFJD23 in *B. pertussis* could not be demonstrated, although HpmB produced in *E. coli* from the same plasmid was able to activate HpmA (Table 3).

Lack of secretion of a HpmA-Fha44 chimera. FHA and HpmA have a 115-residue-long homologous region (9) that was shown to be essential for FHA secretion (28, 35). To investigate whether this HpmA region can functionally replace the homologous FHA region, the corresponding segment of *hpmA* was exchanged for the homologous fragment in *fha44*. The resulting chimeric gene was fused to the OmpA signal peptide-coding sequence, and the plasmid encoding this construct, named pFJD21, was introduced in UT5600 either alone or together with pFJD6 containing *fhaC* or pFJD30 containing *hpmB*. The supernatants and cell sonicates were analyzed by immunoblotting with anti-Fha44 antibodies. Only minute amounts of chimeric Fha44-HpmA were detected in the culture supernatants of the three strains containing pFJD21, irrespective of the presence of *fhaC* or *hpmB* (Fig. 2A, lanes 1 to 3), in contrast to that of wild-type Fha44 produced in the presence of FhaC (Fig. 2A, lane 5), indicating that neither HpmB nor FhaC is able to mediate the secretion of the chimeric protein. The Fha44-HpmA chimera and its precursor, however, were detected in cell sonicates of all strains expressing the hybrid gene (Fig. 2B, lanes 1 to 3).

The hybrid gene was also introduced in *B. pertussis* BPSM and BPGR4 by use of plasmid pBG10, but no anti-Fha44 immunoreactive protein was detected in the culture medium or was cell associated (data not shown), suggesting a rapid proteolytic degradation of the chimera.

Lack of complementation of *hpmB* by *fhaC*. Since HpmB could not complement the lack of FhaC function, it was of interest to know whether FhaC could mediate the secretion and activation of HpmA. Therefore, pFJD20 containing *hpmA* was introduced in *E. coli* UT5600 alone or together with pFJD6, encoding FhaC, or pFJD23, encoding HpmB. No significant hemolytic activity was detected in any of the fractions (supernatants, whole cells, or sonicates) of the strains expressing *hpmA* alone or in *trans* with *fhaC* (Table 3). In contrast, a high level of hemolytic activity was detected in the culture supernatant and on the cell surface of the strain coexpressing *hpmA* and *hpmB*, UT5600(pFJD20, pFJD23) (Table 3). A 10-fold-lower activity was found in sonicates of UT5600 (pFJD20, pFJD23) as compared with that found in whole cells or culture supernatants. This is similar to previous reports showing that secretion and activation of the highly related ShlA hemolysin are tightly coupled (30). Efficient secretion of HpmA was confirmed by the presence of an anti-HpmA immunoreactive protein of the expected size (about 160 kDa) in the supernatant of the strain coexpressing *hpmA* and *hpmB* but not in the culture supernatants of the other strains (data not shown). These observations argue that significant levels of activation and secretion of HpmA are mediated by HpmB but not by FhaC.

We investigated whether ShlB can activate and mediate the secretion of HpmA. Since it has been shown that the in vitro activation of ShlA requires stoichiometric quantities of its accessory protein ShlB (22), a system for high-level expression was chosen. pFJD20 containing *hpmA* was introduced into *E. coli* HMS174(DE3) together with pFJD33 containing *shlB*. As shown in Table 3, the coexpression of *shlB* and *hpmA* resulted in significant levels of hemolytic activity in culture supernatants

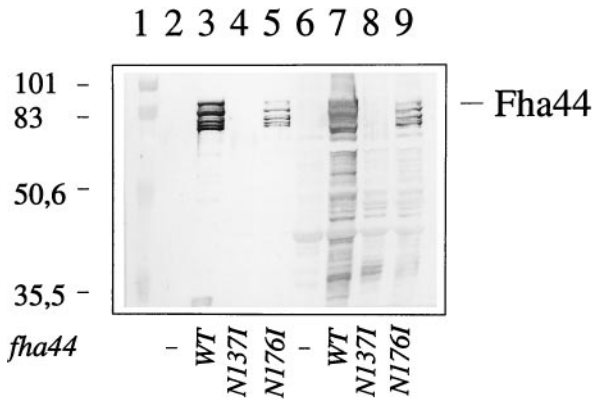

FIG. 3. Secretion of mutant Fha44s in *B. pertussis*. Culture supernatants (lanes 2 to 5) and cell sonicates (lanes 6 to 9) of BPGR4 (lanes 2 and 6), BPGR4 (pFJD9) (lanes 3 and 7), BPGR4(pFJD9-N137I) (lanes 4 and 8), and BPGR4 (pFJD9-N176I) (lanes 5 and 9) were analyzed by immunoblotting with an anti-Fha44 polyclonal antiserum. The same-size volumes of unconcentrated supernatants from liquid cultures whose optical density at 600 nm was about 5.0 were loaded in lanes 2 to 5. Similar amounts of proteins from the clarified sonicates were loaded in lanes 6 to 9. In addition to full-size Fha44, smaller immunoreactive proteins, probably proteolytic degradation products of Fha44, are detected in culture supernatants and sonicate extracts of BPGR4 containing pFJD9 or pFJD9-N176I. Lane 1 contains prestained marker proteins, and the corresponding molecular sizes in kilodaltons are given in the left margin.

and whole cells, demonstrating that the two hemolysin accessory proteins are functionally interchangeable. In contrast, ShlB proved unable to mediate the secretion of Fha44 in *E. coli* (data not shown).

These results prompted us to examine whether FhaC would be able to activate HpmA if produced at a high level. *fhaC* was expressed from the T7 promoter with plasmid pFJD18 in *E. coli* HMS174(DE3), which increased the level of FhaC production (data not shown). However, when pFJD20 expressing *hpmA* was introduced into this strain, significant hemolytic activity was still not detected in the culture supernatants or on whole cells (Table 3), confirming again that FhaC, in contrast with HpmB or ShlB, cannot mediate the activation and secretion of HpmA.

Effect of alterations of the conserved asparagine residues on Fha44 secretion. Two asparagine residues of ShlA (Asn-69 and Asn-109) that lie within the region homologous to FHA have been shown to be essential for ShlA secretion and activation by ShlB (31). Since the hemolysin and FHA accessory proteins are not functionally interchangeable, it was of interest to investigate whether the corresponding asparagine residues of FHA, Asn-137 and Asn-176, respectively, are nevertheless important for its secretion. Each asparagine residue was therefore replaced by isoleucine as described for ShlA. The mutated gene segments were substituted for their wild-type counterpart in pFJD9, which encodes Fha44. The mutant plasmids, pFJD9-N137I and pFJD9-N176I, respectively, were used to transform *B. pertussis* BPGR4. The recombinant strains were analyzed for the presence of Fha44-related proteins in the culture supernatants (Fig. 3). Replacement of Asn-137 by isoleucine totally abolished the secretion of Fha44, whereas the replacement of Asn-176 by isoleucine reduced Fha44 secretion by approximately 80 to 90%, as estimated by enzyme-linked immunosorbent assay. The mutated gene fragments were also introduced into the chromosomal *fhaB* locus by homologous recombination to place the mutation in the context of full-length *fhaB*. The secretion of FHA-N137I and FHA-N176I was affected in a fashion similar to that of their respective Fha44 counterparts (data not shown). This contrasts somewhat with the results obtained for ShlA, where mutations of either residue totally abolished the secretion and activation of the hemolysin. Anti-Fha44 immunoreactive proteins of the sizes of Fha44 and its major proteolytic products were present in cell sonicates of BPGR4(pFJD9-N176I) but in significantly smaller amounts than in cell sonicates of BPGR4(pFJD9) producing wild-type Fha44 (Fig. 3, lane 7 versus lane 9). Only breakdown products were detected in sonicate extracts of BPGR4(pFJD9-N137I) (Fig. 3, lane 8), suggesting that the Fha44 derivatives are rapidly degraded in *B. pertussis*.

Alternatively, the expression of the mutant gene could be inhibited by a feedback mechanism, as described for other secretory proteins (12). We therefore examined whether the presence of either mutant Fha44 interfered with the production of wild-type FHA by using BPSM(pFJD9), BPSM(pFJD9-N137I), and BPSM(pFJD9-N176I), *B. pertussis* strains containing the genes for both full-length FHA and the respective Fha44 variants, each under the control of the *fhaB* promoter. As shown in Fig. 4, FHA was found in similar amounts in the supernatants from all the cultures at the exponential phase (lanes 1, 3, 5, and 7) and at the early stationary phase (lanes 2, 4, 6, and 8). In addition, Fha44 was absent from the culture supernatant of BPSM(pFJD9-N137I), whereas it was secreted by BPSM(pFJD9-N176I), although in small quantities compared with that secreted by BPSM(pFJD9) (lanes 4, 6, and 8). The presence of the secretion-deficient Fha44 variants apparently did not lower the level of production of extracellular FHA, arguing that the mutant Fha44 proteins did not exert feedback inhibition on FHA production.

The lack of cell-associated and extracellular Fha44-N137I may also conceivably be due to an intrinsic instability of this protein compared with Fha44. Since secretion-incompetent proteins in *B. pertussis* are readily degraded (20, 28), this is difficult to assess in this organism. We therefore introduced the mutated genes into the *E. coli* Fha44 secretion system together with pFJD6 containing *fhaC*. Cell-associated anti-

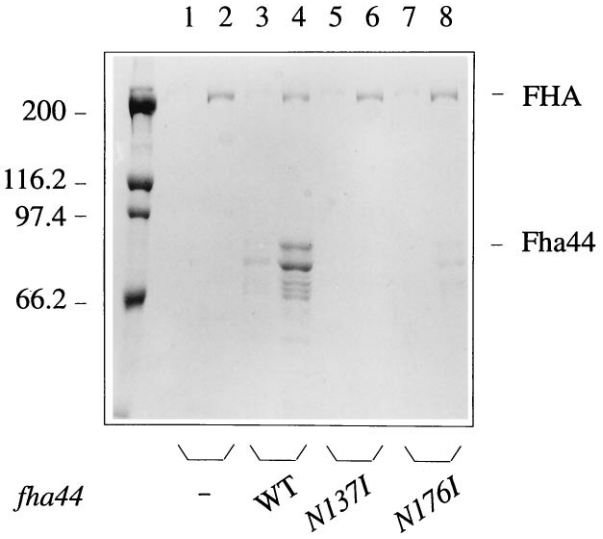

FIG. 4. Secretion of FHA in *B. pertussis* in the presence of mutant Fha44 proteins. Culture supernatants of BPSM(pMMB91) (lanes 1 and 2), BPSM(pFJD9) (lanes 3 and 4), BPSM(pFJD9-N137I) (lanes 5 and 6), and BPSM(pFJD9-N176I) (lanes 7 and 8) collected at the exponential phase (lanes 1, 3, 5, and 7) and at the early stationary phase (lanes 2, 4, 6, and 8) were analyzed by SDS–8% PAGE and Coomassie blue staining. The positions of FHA and Fha44 are indicated. The molecular sizes of the markers in kilodaltons are given in the left margin.

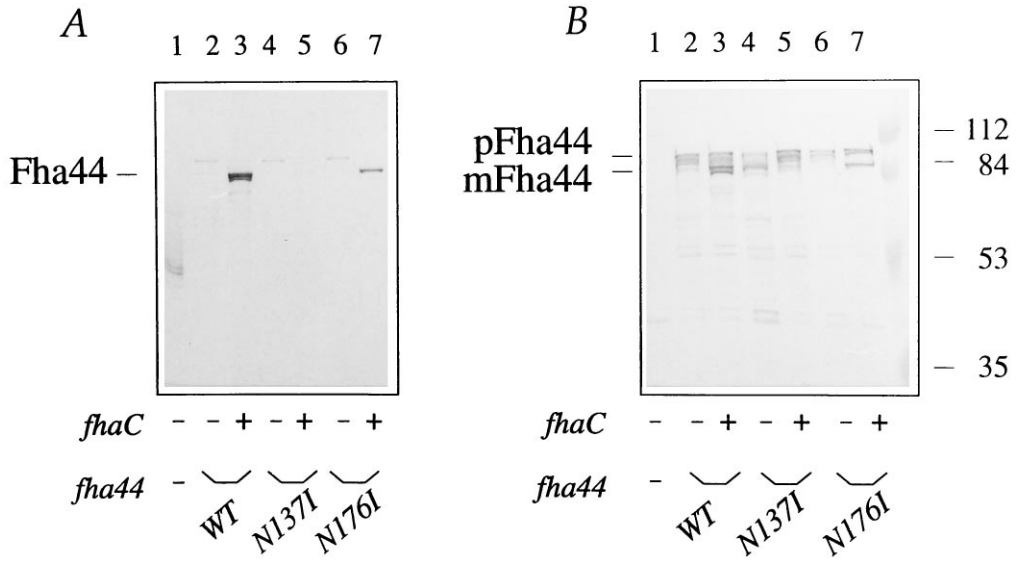

FIG. 5. Secretion of the mutant Fha44 proteins in *E. coli*. Culture supernatants (A) and cell sonicates (B) of UT5600 (lanes 1), UT5600(pFJD11) (lanes 2), UT5600(pFJD11, pFJD6) (lanes 3), UT5600(pFJD38) (lanes 4), UT5600(pFJD38, pFJD6) (lanes 5), UT5600(pFJD39) (lanes 6), and UT5600(pFJD39, pFJD6) (lanes 7) were analyzed by immunoblotting with an anti-Fha44 polyclonal antiserum. The same volumes of supernatants were loaded in each lane of panel A. Similar amounts of proteins from the clarified sonicates were loaded in each lane of panel B. The positions of the Fha44 precursor (pFha44) and processed Fha44 (mFha44) are indicated in the left margin. Prestained marker proteins are shown in the rightmost lane of panel B, and their molecular sizes in kilodaltons are given in the right margin.

Fha44 immunoreactive proteins were present in the cell sonicates of UT5600(pFJD38) expressing *fha44*-N137I and UT5600(pFJD39) expressing *fha44*-N176I, irrespective of the presence of FhaC (Fig. 5B, lanes 4 to 7), in amounts similar to those of Fha44 (Fig. 5B, lanes 2 and 3). Size estimations of the immunoreactive proteins indicated that these strains contained the nonprocessed and mature forms of the Fha44 derivatives together with smaller proteolytic degradation products. In addition, Fha44-N176I was present in the culture supernatants of *E. coli* UT5600(pFJD6, pFJD39), although in significantly smaller amounts than Fha44 secreted by UT5600(pFJD6, pFJD11) (Fig. 5A, lanes 3 and 7). In contrast, no Fha44-N137I was detected in the supernatants of the strains coexpressing *fha44*-N137I and *fhaC* (Fig. 7A, lane 5) or lacking *fhaC* (Fig. 5A, lanes 2, 4, and 6), although small amounts of the Fha44 precursors were found to have leaked out of these cells. The results obtained with *E. coli* are similar to those obtained with *B. pertussis* and indicate that Asn-137 plays a more crucial role than Asn-176 in the secretion of Fha44.

DISCUSSION

Complementation of defects in a secretion machinery with components of a similar secretion apparatus have been attempted in various systems. For the type I secretion system, a high level of functional transcomplementation was suggested to be possible only above a given threshold of sequence similarity between the machineries or between the secretory proteins (11, 17). Heterologous complementation within the type II secretion system met with variable success and was not necessarily reciprocal (8, 18). Likewise, within the family of accessory proteins that make up the type III secretion system, functional transcomplementation was observed when the entire machinery was replaced by a homologous counterpart but was less successful when only individual elements were exchanged (29). The type II and type III machineries are composed of a large number of polypeptides (6, 12, 26). Several of them secrete several unrelated proteins and are thus somewhat promiscuous (14). In *Vibrio cholerae*, for instance, secretion of cholera toxin, the hemagglutinin/protease, and a chitinase depends on the type II Eps machinery (23). In contrast, *B. pertussis* appears to have adapted a specific secretion strategy for each of its secretory proteins. FHA, pertussis toxin, pertactin, and adenylate cyclase/hemolysin each depend on their own secretion machinery rather than upon a general secretory pathway. The high efficiency of the FHA secretion machinery may have evolved at the expense of polyvalency.

Although FHA and the *P. mirabilis* HpmA are two large proteins secreted with the help of similar accessory proteins, FhaC and HpmB, respectively, and share a 115-residue amino-proximal homologous region essential for FHA secretion, these two secretion machineries are not interchangeable. It is possible that the two systems are phylogenetically too distant. The level of strict identity of the outer membrane proteins FhaC and HpmB is only 16%, although they are similar throughout their entire length (35). On the other hand, the amino-proximal regions of FHA and HpmA have about 47% sequence identity over 115 residues (19). In contrast, HpmB has 55% identity with ShlB, the accessory protein involved in the secretion of the *S. marcescens* ShlA hemolysin, and the two hemolysins contain 46.7% identical residues (33). ShlB was previously shown to activate HpmA in vitro (22), and our results indicate that ShlB can functionally replace HpmB in vivo both for secretion and activation of HpmA.

Whereas the only role demonstrated to date for FhaC is linked to the secretion of FHA, ShlB and HpmB are involved in both secretion and activation of their cognate hemolysins (22, 30). Both activaties of ShlB have not been dissociated yet, and the activation and secretion of the hemolysins appear to be tightly coupled (5, 31). To date, there is no evidence to suggest that FHA undergoes an activation step. This may be one of the fundamental differences between FhaC on the one hand and ShlB and HpmB on the other hand. FhaC may be unable to activate HpmA, and it is reasonable to hypothesize that the accessory proteins of the Ca^{2+}-independent hemolysins possess an uncharacterized modifying activity absent in FhaC.

Likewise, secretion of Fha44 cannot be mediated by HpmB, suggesting that the conserved region of FHA is not recognized by HpmB. This is somewhat more surprising, since the domain indispensable for hemolysin activation and secretion lies precisely in the region of homology shared by FHA and the hemolysins (31). Even the replacement of the amino-proximal region of FHA with that of HpmA did not restore HpmB-mediated secretion, suggesting that it is not the sole secretion determinant of the protein.

Among the amino acid residues previously shown to be essential for the activation or secretion of ShlA, two asparagine residues are conserved in FHA (Table 1). Interestingly, site-directed mutagenesis experiments described here indicate that both are involved in, but only the first one is essential for, FHA secretion, whereas both appeared to be equally important in ShlA secretion and activation, and only the second one is conserved in HMWA. Activation of ShlA is brought about by a direct interaction with ShlB and is likely to result from a modification, the nature of which has to date remained elusive (31). Several observations argue against a simple conformational change, and although the presence of a covalent adduct has not been shown, the putative modification appears to be irreversible. It takes place in the amino-proximal region of ShlA and confers upon the hemolysin the ability to adhere to erythrocytes (22). Whether one of the two invariant asparagine residues is actually the site of modification has not yet been demonstrated.

The absence of FhaC or alterations of FHA which impede its secretion apparently result in extensive proteolytic degradation of the secretory protein in *B. pertussis*. The fact that the Fha44 mutants are found to be cell associated in *E. coli* argues against a major defect in stability and in favor of a defect in secretion due to the mutations. In *B. pertussis*, the stress due to the presence of secretion-incompetent proteins in the cell envelope might conceivably activate an envelope protease(s), as recently described for *E. coli* (7), or alternatively result in a feedback inhibition of *fhaB* expression. Our results show that the nonsecreted FHA derivatives undergo extensive proteolytic degradation in *B. pertussis*. Indeed, accumulation of periplasmic intermediates does not seem to occur in *B. pertussis* (20, 28), suggesting that it has an efficient envelope proteolytic machinery. In addition, the partial proteolytic degradation even of secretion-competent FHA suggests that it might move across the cell envelope in an unfolded form, and thus a significant portion would remain sensitive to proteases before secretion is completed.

Obviously, FHA has evolved in a modular fashion by the juxtaposition of several functional blocks into a secretion-competent protein. The accessory protein FhaC may have adapted to fit specific requirements of its cognate secretory partner. In addition to the putative interaction between FhaC and the amino-proximal region of FHA, the 150-kDa carboxy-terminal extension of the FHA precursor appears to play an important role in secretion (28). Furthermore, it is not excluded that additional accessory proteins might be involved in the secretion of FHA. Such additional accessory proteins are required to mediate the secretion of the HMWA adhesins in *H. influenzae* (3). However, no homologs of these proteins, named HMWC, or any other accessory factors necessary for the secretion of FHA have been isolated yet, but it is quite likely that the secretion of FHA makes use of several general cellular factors that remain to be identified.

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