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The RTX Cytotoxin-Related FrpA Protein of *Neisseria meningitidis* Is Secreted Extracellularly by Meningococci and by HlyBD$^+$ *Escherichia coli*†

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Neisseria meningitidis **produces proteins (FrpA and FrpC) related to the RTX cytotoxin family. In meningococcal strain FAM20 these proteins were both localized in the outer membrane and secreted into the extracellular medium. An *Escherichia coli* strain with wild-type hemolysin secretion genes *hlyB* and *hlyD* and containing a cloned *frpA* gene secreted FrpA, whereas an isogenic *hlyBD* mutant strain did not. Low-stringency DNA hybridization revealed *hlyBD*-like sequences in *N. meningitidis* FAM20, suggesting that a similar RTX secretion system exists in meningococci. Structural features found at the C termini of other RTX proteins and thought to be important for their secretion were also found at the C terminus of FrpA. The secretion of FrpA from *E. coli* by heterologous RTX transport proteins further demonstrates the relation of the FrpA protein to RTX toxins.**

Neisseria meningitidis is an obligate human pathogen responsible for outbreaks of meningitis and septicemia, especially in underdeveloped areas of the world. Although meningococci are not thought not to produce exotoxins (6), *N. meningitidis* FAM20 was recently shown to produce proteins (FrpA [122 kDa] and FrpC [198 kDa]) with DNA and predicted protein sequence similarities to the RTX family of cytotoxins (32, 33). The functions of the meningococcal proteins have not been determined. Members of the RTX family are widespread among gram-negative bacteria and include proteins that function as either cytolysins or proteases (35).

In contrast to secretion mediated by the *Escherichia coli sec* genes and involving N-terminal secretion signals, most RTX proteins are secreted from the bacterial cell by a unique secretion apparatus that depends on signals found at the C terminus of each RTX protein (31, 35). The secretion machinery is conserved by bacteria expressing RTX proteins and consists of three membrane proteins that mediate transport of the protein from the cytoplasm directly to the extracellular space. In the case of the *E. coli* hemolysin (HlyA), the proteins responsible for the secretion of the hemolysin are HlyB and HlyD (9) and TolC (34). The *hlyB* and *hlyD* genes are cotranscribed with *hlyC* and *hlyA* (17, 36), while *tolC* is located elsewhere on the chromosome (34). In some cases the conservation of HlyB- and HlyD-like proteins has been demonstrated by the ability of these proteins to mediate secretion of heterologous RTX proteins. For example, the *E. coli* HlyB and HlyD proteins are able to secrete from *E. coli* the RTX proteins of *Pasteurella haemolytica* (LktA) (3), *Bordetella pertussis* (CyaA) (26), and *Actinobacillus pleuropneumoniae* serotype 1 (HlyIA) (13). Mutational analysis of the C-terminal 48 amino acids of *E. coli* HlyA revealed several structural features that are conserved among other RTX proteins and that are thought be important in interaction with the HlyB-HlyD-TolC apparatus (16, 31). An interesting exception to the secretion of RTX proteins is the *Actinobacillus actinomycetemcomitans* leukotoxin (LktA), which possesses the C-terminal secretion features but remains associated with the cell surface (19, 21).

This report demonstrates that meningococcal FrpA and FrpC are both secreted and associated with the outer membrane. Secretion of the meningococcal FrpA protein is mediated in *E. coli* by the hemolysin secretion apparatus, underscoring the relatedness of FrpA with RTX proteins. Comparisons of the C-terminal structure of FrpA with that of other RTX proteins revealed common features known to be important for secretion by RTX transport proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. For maximal expression of meningococcal RTX-related proteins FrpA and FrpC, meningococci were grown in chemically defined medium (CDM) in acid-washed glassware as previously described (38). *E. coli* strains were grown in Luria-Bertani broth (24) supplemented with 50 μg of ampicillin and/or 30 μg of chloramphenicol per ml as needed.

Protein gel conditions and immunoblotting. Cultures of *E. coli* strains grown overnight in the presence of appropriate antibiotic selection or *N. meningitidis* FAM20 grown in CDM were centrifuged and then filtered through 0.20-μm Acrodisc filters (product no. 4192; Gelman, Ann Arbor, Mich.) to remove residual bacterial cells. In addition, meningococcal culture supernatants were centrifuged at 100,000 × *g* for 2 h to ensure the removal of naturally elaborated membrane blebs. Trichloroacetic acid (TCA) precipitates of these supernatants were prepared as previously described (9). Total cell protein samples were prepared by collecting the cells contained in 500 μl of overnight *E. coli* culture or in 500 μl of *N. meningitidis* grown in CDM to a density of 100 Klett units. Cells were harvested by a 5-min centrifugation in a microcentrifuge and then were resuspended in 100 μl of 1× sodium dodecyl sulfate (SDS)-

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† This paper is dedicated to the memory of Phil Bassford, whose discussions regarding this work were invaluable.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference
Bacterial strains		
N. meningitidis FAM20	*frpA*$^+$ *frpC*$^+$	7
E. coli		
WAM582	*hlyCABD*$^+$ *tolC*$^+$	36
WAM716	*hlyBD*$^+$ *tolC*$^+$	10
WAM716(pUNCH216)	*hlyBD*$^+$ *tolC*$^+$ *frpA*$^+$	This study
DH1	*tolC*$^+$	24
DH1(pUNCH216)	*tolC*$^+$ *frpA*$^+$	This study
Plasmids		
pUNCH216	*frpA*$^+$	33
pWAM716	*hlyBD*$^+$	10

polyacrylamide gel electrophoresis solubilization buffer (14). Total and outer (i.e., Sarkosyl-insoluble) membrane proteins were prepared as previously described (12, 37). *N. meningitidis* outer membrane blebs were isolated following extraction of cells in lithium chloride buffer and agitation with glass beads (22). Protein samples were electrophoresed in SDS–7.5% polyacrylamide gels and transferred to NitroPlus membranes (Micron Separations, Inc., Westboro, Mass.). Immunoblotting was done as previously described (2, 14), with the monoclonal antibodies (MAbs) 9D4 (anti-*B. pertussis* adenylate cyclase toxin [CyaA]; gift of Erik Hewlett), which binds an epitope in the nine-amino-acid repeat region of RTX proteins (23), and 15D6 (anti-gonococcal protein IA [11]) at a 1:1,000 dilution.

DNA hybridizations. High-stringency (1) and low-stringency (30) DNA hybridizations were done as previously described, using as a probe the plasmid pWAM716 (10) labeled by random priming with ^{32}P (8).

RESULTS

Localization of RTX-homologous proteins in *N. meningitidis* FAM20. The recently described meningococcal RTX toxin homologs FrpA and FrpC were originally identified as constituents of meningococcal outer membrane preparations. Since most RTX toxins are secreted proteins (with the notable exception of the *A. actinomycetemcomitans* LktA leukotoxin, an outer membrane protein), we asked whether the meningococcal RTX homologs also were secreted. We prepared whole-cell, total membrane, outer membrane, cytoplasmic, lithium chloride-extracted membrane bleb, and secreted fractions from *N. meningitidis* FAM20. As seen previously, the meningococcal proteins were found in preparations containing outer membrane proteins (Fig. 1A, lanes 2 and 3). In addition, FrpA and FrpC were found in filtered culture supernatants that had undergone ultracentrifugation to remove naturally elaborated membrane blebs (Fig. 1A, lane 6). To verify that the proteins detected in Fig. 1A, lane 6, were in fact secreted and were not present because of contaminating membrane fragments, the filter shown in Fig. 1A was probed with MAb 15D6. This MAb was generated against gonococcal porin protein IA but also recognizes the meningococcal porins (11), the most abundant proteins in the meningococcal outer membrane. Binding of 15D6 by fractions containing outer membrane proteins was easily detectable, but we were unable to detect any binding by TCA-precipitated FAM20 culture supernatants (Fig. 1B). A similar pattern of binding was seen with MAb 4B12 (Milan Blake, The Rockefeller University, New York, N.Y.), which binds outer membrane opacity proteins (data not shown).

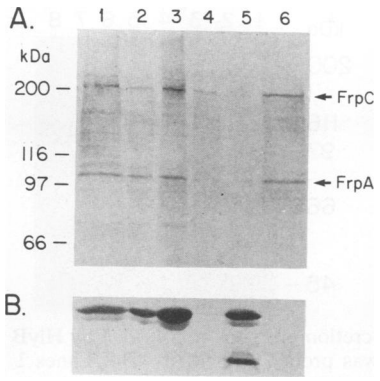

FIG. 1. Localization of RTX-related proteins in *N. meningitidis* FAM20. (A) The immunoblot was probed with MAb 9D4. (B) The immunoblot from panel A was probed with antiporin MAb 15D6. Samples used were solubilized whole-cell proteins (lane 1), total membranes (lane 2), outer membranes (lane 3), cytoplasmic proteins (lane 4), lithium chloride-extracted outer membrane blebs (lane 5), and TCA-precipitated culture supernatant (lane 6). Lane 1 contained the amount of protein from approximately 5×10^8 cells, lanes 2 through 5 contained 20 μg of protein, and lane 6 contained the amount of protein precipitated from 1.0 ml of filtered culture supernatant.

Therefore, as with most other RTX proteins, FrpA and FrpC were secreted into the medium by FAM20. Interestingly, lithium chloride-extracted membrane blebs did not contain an appreciable amount of FrpA and FrpC (Fig. 1A, lane 5), although the porins were clearly represented (Fig. 1B, lane 5).

Secretion of FrpA by *E. coli* expressing HlyB and HlyD. The structures at the C termini of RTX toxins that serve as secretion signals, as well as the proteins required for their secretion, have been conserved among the bacteria secreting RTX proteins. This conservation is demonstrated by the ability of some of the transport proteins to mediate secretion of heterologous RTX toxins (3, 13, 26). To investigate whether the C-terminal toxin structures required for recognition by the RTX transport proteins were also conserved in the meningococcal FrpA protein, we tested the ability of the *E. coli* HlyB and HlyD proteins to direct secretion of FrpA into the extracellular space. All strains used in these experiments expressed the TolC protein. WAM582 expresses the entire *hly* operon (*hlyCABD*) and secretes an active hemolysin into the medium (Fig. 2, lane 1). HlyA is not processed during the secretion process and remains the same size as its primary translation product (Fig. 2, lane 5). To test FrpA secretion, pUNCH216 was introduced into WAM716, which expresses the HlyB and HlyD proteins (but not HlyA or HlyC). FrpA was secreted into the medium by this strain (Fig. 2, lane 3). The size of the secreted protein (105 kDa), however, was somewhat smaller than that observed in samples taken from solubilized whole cells and which corresponded to the 122-kDa size predicted by the *frpA* open reading frame (Fig. 2, lane 7). Whether this was due to cleavage during secretion or subsequent processing was not known from this experiment, but the 105-kDa protein was also seen in meningococcal preparations (Fig. 1A, lanes 1 to 4 and 6). FrpA was not secreted into the culture supernatant by DH1 (isogenic with WAM716 but lacking HlyB and HlyD) (Fig. 2, lane 4), although its expression in DH1 appeared to be equivalent to that in WAM716 (Fig. 2, lane 8 versus lane 7).

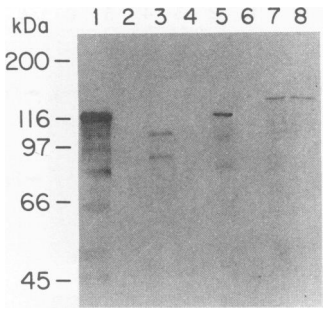

FIG. 2. Secretion of FrpA from *E. coli* by HlyB and HlyD. The immunoblot was probed with MAb 9D4. Lanes 1 through 4 contained TCA-precipitated proteins from filtered culture supernatants (1.0 ml each); lanes 5 through 8 contained solubilized whole-cell proteins. Samples were WAM582 (lanes 1 and 5), WAM716 containing pBluescript SK− (lanes 2 and 6), WAM716 containing pUNCH216 (lanes 3 and 7), and DH1 containing pUNCH216 (lanes 4 and 8).

Lack of membrane association of FrpA in *E. coli*. Since FrpA was present in both outer membrane and secreted fractions of *N. meningitidis* FAM20, we asked whether the FrpA protein could be inserted into *E. coli* membranes in either the presence or absence of HlyB and HlyD. The isogenic strains DH1(pUNCH216) and WAM716 (pUNCH216), which differed only in their expression of HlyB and HlyD, were used for these experiments. Each was separated into secreted, whole-cell, total membrane, outer membrane, and cytoplasmic fractions. As before, only WAM716 was able to secrete FrpA from the cell (Fig. 3, lane 1 versus lane 6). Unlike in FAM20, FrpA was not membrane associated in either *E. coli* strain (Fig. 3, lanes 3, 4, 8, and 9). Interestingly, in both cytoplasmic fractions the predominant form of FrpA was the 105-kDa protein (Fig. 3, lanes 5 and 10). This indicated that the conversion of the 122-kDa protein to the 105-kDa form of FrpA was not dependent on secretion, since cleavage occurred even in the secretion-deficient strain DH1.

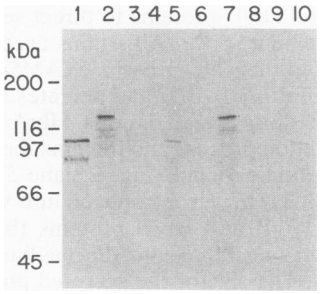

FIG. 3. Localization of FrpA in secretion-proficient and secretion-deficient *E. coli* strains. The immunoblot was probed with MAb 9D4. Lanes 1 through 5 were derived from the secretion-proficient strain WAM716, and lanes 6 through 10 were derived from the isogenic but secretion-deficient strain DH1; both strains contained pUNCH216. Samples used were TCA-precipitated culture supernatant (lanes 1 and 6), solubilized whole-cell proteins (lanes 2 and 7), total membranes (lanes 3 and 8), outer membranes (lanes 4 and 9), and cytoplasmic proteins (lanes 5 and 10). Lanes 1 and 6 contained the amount of protein precipitated from 1.0 ml of filtered culture supernatant, lanes 2 and 7 contained the amount of protein from approximately 5×10^8 cells, and lanes 3 through 5 and 8 through 10 contained 20 μg of protein each.

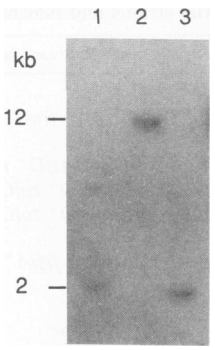

FIG. 4. Identification of *hlyBD*-like sequences in FAM20, showing low-stringency DNA hybridization using ^{32}P-labeled pWAM716 (*hlyBD*$^+$) as a probe against FAM20 chromosomal DNA digested with *Ava*I (lane 1), *Cla*I (lane 2), and *Sau*3AI (lane 3). The filter was exposed to film for 4 days.

Presence of HlyBD-like sequences in *N. meningitidis* FAM20. The genes homologous to the *E. coli hlyB* and *hlyD* genes are conserved in bacteria expressing RTX proteins (35). We used low-stringency DNA hybridization to determine if FAM20 also had homologs of the *hlyBD* genes. The *hlyBD* genes cloned on pWAM716 were used as a probe against *N. meningitidis* FAM20 chromosomal DNA digested with *Ava*I, *Cla*I, and *Sau*3AI. In each digest a single restriction fragment hybridized with the *hlyBD* probe, indicating that sequences homologous to *hlyB* or *hlyD* were present in FAM20 (Fig. 4). *E. coli* DH1 (HlyBD$^-$) was used as a negative control and showed no hybridization with the *hlyBD* genes (data not shown).

Analysis of C-terminal features found in FrpA. Stanley et al. previously identified by mutational analysis several features at the C terminus of *E. coli* HlyA that were important for its efficient secretion from *E. coli* and made comparisons of these structures with similar ones found at the C termini of other RTX proteins (31). In their comparative analysis, Stanley et al. used the sequence of the *A. actinomycetemcomitans* leukotoxin that was reported by Lally et al. (21). This sequence was found to be in error by Kraig et al. (19). We have therefore used the sequence reported by Kraig et al. for purposes of comparison with FrpA.

The three predominant functional domains identified by Stanley et al. (31) were (i) an eight-amino-acid stretch containing five hydroxylated residues at the extreme C terminus of the HlyA protein, (ii) a region of 13 uncharged amino acids present 16 to 28 residues before the C terminus, and (iii) a charged, amphipathic helix upstream of the uncharged region. Analysis of the C-terminal 52 amino acids of FrpA revealed several features in common with *E. coli* HlyA (Fig. 5A). For comparison, both HlyA (secreted protein) and the *A. actinomycetemcomitans* LktA (outer membrane associated) are shown. FrpA contained an area of hydroxylated residues at its C terminus, although the number of hydroxylated residues was fewer than in either HlyA or LktA. Two of the C-terminal eight residues of FrpA were hydroxylated, as opposed to five for HlyA and three for LktA (Fig. 5A). A 26-amino-acid stretch of uncharged amino acids preceding the hydroxylated residues of FrpA was present (residues −18 to −43 relative to the C terminus) (Fig. 5A) in a position similar to that of the uncharged regions of both HlyA and LktA. In fact, the C terminus of FrpA was highly devoid of charge, with the Asp residue at position −17 being the only charged amino acid in the

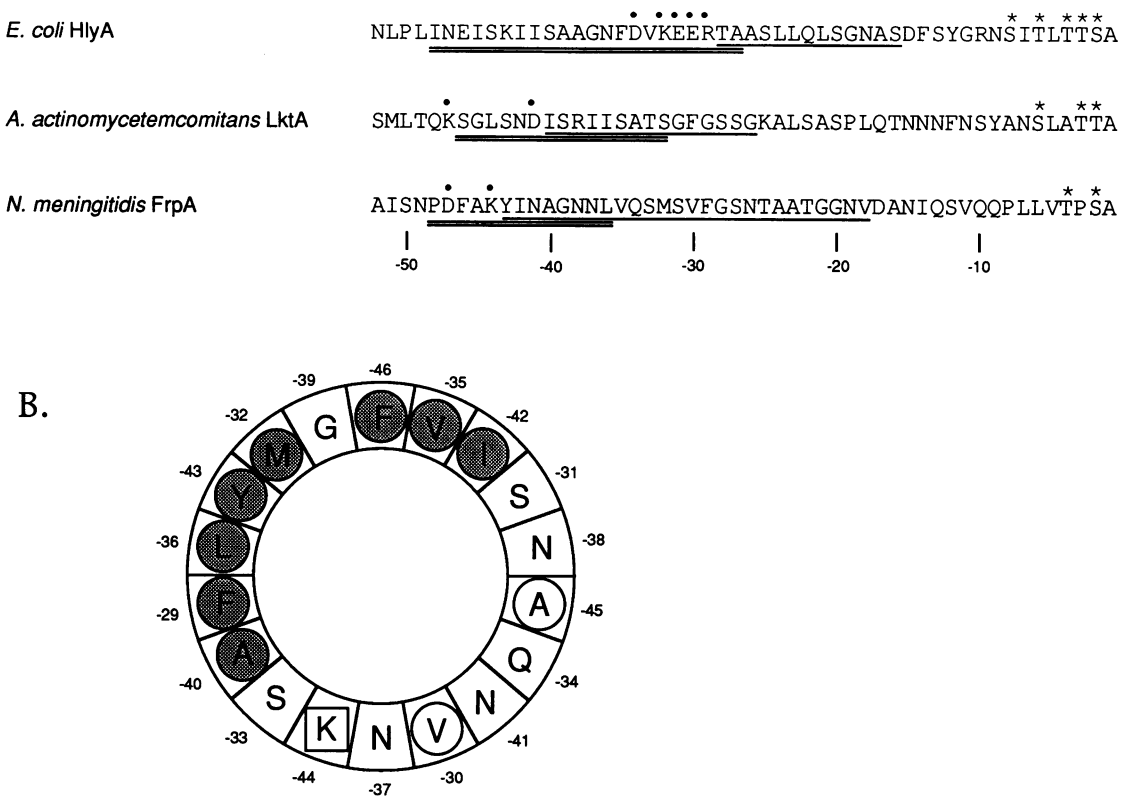

FIG. 5. Diagram of the C terminus of FrpA, showing conserved structural features. (A) Shown are the C-terminal 52 amino acids of *E. coli* HlyA from pHly152 (15), *A. actinomycetemcomitans* LktA (19), and *N. meningitidis* FrpA (33). Amino acids are numbered relative to the C terminus of the protein. Features indicated are (i) small, hydroxylated residues near the C terminus (*), (ii) the uncharged region preceding the hydroxylated residues (single underline), (iii) the small cluster of charges upstream of the uncharged region (164), and (iv) a potential amphipathic helix-forming region predicted by the program of Margalit et al. (25) (double underline). (B) Helical wheel representation (29) of the potential amphipathic helical region (residues −46 to −29) of the FrpA C terminus. Hydrophobic residues (circles) and charged residues (squares) are indicated. The residues on the hydrophobic face of the potential amphipathic helix are shaded.

C-terminal 46 amino acids of the protein. The area preceding the uncharged region of FrpA was predicted by the program of Margalit et al. (25) to be an amphipathic helix, the core of which extends from residues −36 to −48 (Fig. 5A). This too was at a location similar to that found in both HlyA and LktA (31). The potential amphipathic helical nature of the region extending from residues −46 to −29 can be represented as a helical wheel (29), showing the location of hydrophobic residues along one face of the helix (Fig. 5B).

DISCUSSION

We have shown that *N. meningitidis* FAM20 produces proteins related to the RTX family of exoproteins (32, 33). The previous work identified multiple nine-amino-acid repeats in FrpA that are essentially identical to those found in all other RTX family members (35) and also showed that a MAb specific for RTX proteins bound both FrpA and FrpC (33). Here we demonstrate that FrpA and FrpC were both secreted and membrane bound in FAM20.

Most RTX proteins are secreted into the medium, presumably by a mechanism involving no periplasmic intermediate (9, 18). In the case of the *E. coli* hemolysin (HlyA), this is achieved through the action of three proteins, HlyB, HlyD, and TolC (9, 34). These proteins act in concert to recognize the C terminus of HlyA and to direct its secretion outside the cell. HlyB- and HlyD-like proteins are also found in other RTX toxin-producing bacteria (35). Furthermore, the C termini of the various RTX toxins show structural similarity, implying a common mechanism of secretion (31).

Neither FrpA nor FrpC possesses a typical N-terminal signal sequence (32, 33). Lack of an N-terminal signal sequence is characteristic of all RTX proteins, which are secreted through a unique pathway (35). FrpA apparently also possesses the structural features necessary to be secreted by the *E. coli* secretion apparatus (HlyB, HlyD, and TolC). Secretion was dependent on the *E. coli* HlyB and HlyD proteins, since secretion did not occur in their absence. However, in contrast to what was observed in *N. meningitidis*, FrpA was not directed into the membrane in *E. coli*. It is not clear what allows FrpA (and FrpC) to maintain an association with the meningococcal outer membrane and also to be secreted. Analysis of the C terminus of FrpA reveals features common to other RTX proteins. This analysis also applies to the FrpC protein, since FrpA and FrpC differ by only a single amino acid in the C-terminal 121 amino acids (32, 33). The number of hydroxylated residues found in the C-terminal eight amino acids of FrpA (two) is fewer than

the number in either HlyA (five) or LktA (three) but is identical to the number found in five other RTX proteins (31). FrpA also has the ability to form an amphipathic helix centered at 36 to 48 residues preceding the C terminus (Fig. 5), a position where similar structures are found in the secreted RTX proteins and in the cell-associated LktA (31). The net charge of the final 25 amino acids of RTX proteins is between −1 and +1 (31). FrpA has a net charge of −1, similar to that of both secreted and cell-associated RTX proteins. It appears, therefore, that the C-terminal structures identified by Stanley et al. (31) are found in both secreted and cell-associated RTX proteins as well as FrpA and FrpC. It may be that extracellular or membrane localization of RTX proteins is due to subtle differences in RTX transport proteins (e.g., HlyB and HlyD), as suggested by Lally et al. (20).

FAM20 probably has homologs of the *E. coli hlyB* and/or *hlyD* genes, as determined by low-stringency DNA hybridization. It is not surprising that FAM20 would have such genes, given their conservation in other bacteria that secrete RTX proteins. The *hlyB* and *hlyD* homologs are apparently not closely linked to the *frpA* or *frpC* genes, as determined by DNA hybridization and by limited sequence data for the regions surrounding the *frpA* and *frpC* loci (data not shown). Although *hlyBD* homologs are often directly linked to the *hlyA*-like genes, in *A. pleuropneumoniae* serotype 5 the *hlyB* and *hlyD* genes are found elsewhere on the chromosome (4).

Unlike the other RTX proteins, FrpA may undergo processing. It is uncertain whether the many bands seen in immunoblots of the meningococcal proteins (Fig. 1) represent nonspecific proteolysis or processing that is essential for either protein function or secretion, since the functions of the meningococcal RTX homologs are unknown. FrpA may have an autoproteolytic function similar to that of the neisserial immunoglobulin A protease (28), a function that may be necessary for activation of the protein. It is interesting that some of the RTX proteins (e.g., *Erwinia chrysanthemi prtB* and *prtC* proteins and *Serratia marcescens* extracellular metalloprotease) are proteases (5, 27). It is also possible that the smaller protein species result from other factors, such as the use of multiple promoters or alternate sites of translation initiation. Further studies are needed to determine whether there is proteolytic cleavage of the FrpA protein, the complete mechanism of secretion of RTX proteins from meningococci, and the functional significance of these proteins.

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