The Neisseria meningitidis Outer Membrane Lipoprotein FrpD Binds the RTX Protein FrpC†

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At conditions of low iron availability, Neisseria meningitidis produces a family of FrpC-like, type I-secreted RTX proteins of unknown role in meningococcal lifestyle. It is shown here that iron starvation also induces production of FrpD, the other protein expressed from a gene located immediately upstream of the frpC gene in a predicted iron-regulated frpDC operon. We found that FrpD is highly conserved in a set of meningococcal strains representative of all serogroups and does not exhibit any similarity to known sequences of other organisms. Subcellular localization and [3H]palmitic acid exhibit any similarity to known sequences of other organisms. FrpD is highly conserved in a set of meningococcal outer bacterial membrane.

loci-encoded accessory lipoprotein that could be linked to that of the RTX protein FrpC, because FrpD was found to bind the amino-proximal portion of FrpC (first 300 residues) with very high affinity (apparent $K_d$ ~0.2 nM). These results suggest that FrpD represents an rtx loci-encoded accessory lipoprotein that could be involved in anchoring of the secreted RTX protein to the outer bacterial membrane.

Neisseria meningitidis is a Gram-negative bacterium colonizing the nasopharynx of about 10% of healthy humans. Meningococci can, however, occasionally also be pathogenic and traverse the mucosal epithelia to reach the bloodstream, eventually cross the blood-brain barrier, and cause rapidly progressing septicemia and/or meningitis. Sporadic outbreaks or epidemics of invasive meningococcal disease (i.e. in Sub-Saharan Africa), indeed, still remain an important cause of mortality and morbidity throughout the world (1–4).

The molecular basis of meningococcal virulence remains difficult to analyze, because human colonization and invasive disease are not adequately reproduced in current animal models. Several traits potentially required for virulence of meningococci have, however, been identified, including production of a capsule conferring resistance to serum, secretion of an IgA protease, the high antigenic variability of pili and of several non-fimbrial adhesins, and the presence of several iron acquisition systems (5).

Under conditions of limited iron availability, N. meningitidis was shown to produce RTX (repeat in toxin) family proteins secreted through the type I pathway, the so-called FrpC-like proteins (6–10). These are characterized by the presence of a variable number of carboxyl-proximal glycine and aspartate-rich repetitions of a nonapeptide RTX consensus motif (L/I/F/XGGXG(D/N)DX (11). While the biological activity of meningococcal FrpC-like proteins remains unknown, a number of other RTX proteins was already shown to act as exotoxins and to play an important role in virulence of Gram-negative pathogens of the genera Escherichia (HlyA), Actinobacillus (LtxA, Apx proteins), Bordetella (CyaA), Vibrio (RtxA), or Mannheimia (LkA) (12–17). While HlyA, CyaA, and LtxA, and some Apx toxins exhibit pore-forming activity capable of perturbing bacterial activities of leukocytes by membrane permeabilization and colloid-osmotic cell lysis (at high concentrations), the Bordetella CyaA also harbors an adenylate cyclase enzyme activity penetrating myeloid phagocytes, paralyzing them by cAMP intoxication, and Vibrio cholerae RtxA causes actin cytoskeleton perturbation and cell morphology changes by catalyzing cross-linking of actin subunits (12–17). Furthermore, RTX proteins were found to exhibit lipase, protease, bacteriocin, or nodulation-inducing activities or form S-layers on bacterial cell surface (18–22).

Genetic organization of most of the characterized rtx determinants is similar to that of the locus accounting for production of the E. coli α-hemolysin (HlyA). This consists of an operon of four structural genes (hlyCABD) encoding the RTX protein itself (HlyA), an acyltransferase activating the toxin by post-translational fatty acylation (HlyC), and two inner membrane components of the type I secretory apparatus (HlyB and HlyD), required for extracellular secretion of the toxin (23–28). Some rtx loci also contain a gene for the outer membrane ToLC-like channel component of the type I secretion machinery (29), whereas the loci encoding the RTX proteases contain a gene for an inhibitor of the respective protease, in place of the acyltransferase gene (30–32). In N. meningitidis, however, neither the gene for the acyltransferase, nor the genes for components of the secretion machinery are present within the frpC locus. Instead, the frpC gene is preceded by an open reading frame, orfI (GenBankTM acc. no. L06299) encoding a hypothetical protein of unknown function (7) that does not exhibit similarity to any other known protein sequence. Moreover, the

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1 The abbreviations used are: RTX, repeat in toxin; BCIP, 5-bromo-4-chloro-3-indolylphosphate; CBD, chitin binding domain; Frp, Fe-regulated protein; IPTG, isopropyl-β-D-thiogalactopyranoside; OMP, outer membrane protein; DTT, dithiothreitol; CHAPS, 3-[3-cholamidopropyl(dimethylammonio)-1-propanesulfonic acid; PBS, phosphate-buffered saline; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; BSA, bovine serum albumin.
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genetic organization suggests that the orf1 and frpC genes form an operon transcribed from an iron-regulated promoter located ~300-bp upstream of orf1, where a predicted binding site for the ferric uptake regulator protein Fur could be identified (7).

Here we show that Orf1 is an outer membrane lipoprotein that binds the N-terminal portion of FrpC with high affinity and represents, thereby, a new class of accessory proteins of the RTX determinants. We propose to call it FrpD.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—For production of the entire FrpD (FrpD271) and of its shorter FrpD250 variant in *E. coli*, the frpD271 and frpD250 open reading frames of *N. meningitidis* 10/96 (8) were amplified by PCR and cloned as NdeI-BamHI fragments in the pTT7-7 vector (33), yielding pT7-7-frpD271 and pT7-7-frpD250. The frpD271 and frpD250 reading frames were next transferred into pET28b (Novagen) and fused in-frame with the sequence encoding six consecutive His residues to obtain pET28/frpD271 and pET28/frpD250 plasmids for production of FrpD271-6xHis and FrpD250-6xHis proteins. Similarly, pSU39/frpD271, derived from pET28/frpD271, by recombining frpD271-6xHis into pSU39 (34), pSU39frpD419, and pSU39frpD419-6xHis, for production of FrpD419, or inserting the frpD271 and frpD250 reading frames as Xbal fragments into pTYB2frpC (8). The pTYB2frpC-XAY plasmids, where X and Y stand for the numbers of the first and of the last amino acid residues of the FrpC segment deleted in the given construct, were prepared by PCR mutagenesis and/or by using the naturally occurring restriction sites in *frpD* alleles and the absence of undesired mutations in PCR-amplified DNA were systematically verified by DNA sequencing, and plasmid maps with complete sequences will be provided upon request.

**Cloning and Sequencing of frpD Alleles**—1186-bp long PCR products comprising the frpD alleles were amplified with the orf1-frpC primer pair (8) from chromosomal DNA of nine representative meningococcal isolates in *N. meningitidis* P1/75K and in *N. gonorrhoeae* M45. Construction of plasmids and the absence of undesired mutations in *frpD*-amplified DNA were systematically verified by DNA sequencing, and plasmid maps with complete sequences will be provided upon request.

**Cultures of Meningococci**—The *N. meningitidis* isolate 10/96 (8) was grown on GCB agar plates (Difco) at 37°C in a humidified 5% CO2 atmosphere for 10 h. Bacteria were washed and resuspended with RPMI (without fetal calf serum) for 10 h until OD600 reached 0.2–0.3 (maximal viability), 100 nCi/ml [3H]palmitic acid (Amersham) were resuspended in 1 ml of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl buffer (TN buffer), and disrupted by sonication (20 s, 50% amplitude), followed by centrifugation at 4000 rpm for 10 min. The lysate was overlaid on a sucrose cushion (0.4% of 30% and 0.3% of 65% w/w sucrose) and centrifuged for 2 h at 40,000 rpm in a Sorvall TH-641 rotor for 40 h at 10°C. Membrane suspension was recovered at the equilibrium at 59,000 rpm for 40 h at 10°C in a Sorvall TST 60.4 rotor, and 200-μl gradient fractions were analyzed for the presence of the FrpD-6xHis and LamB proteins by immunoblotting with anti-polyhistidine monoclonal antibody (Sigma) and rabbit polyclonal serum against LamB (gift of A. Charbit), respectively. NADH oxidase activity was determined as previously described (35).
RESULTS

FrpD Is Conserved among Clinical Isolates of N. meningitidis—We have previously examined the distribution of frpD alleles in a set of 65 N. meningitidis isolates and shown that the frpD gene was present in all 38 examined invasive isolates as well as in 24 of 27 noninvasive isolates, respectively (8). Conservation of the frpD sequence was, therefore, analyzed for nine alleles from clinical isolates representing all meningococcal serogroups, and the deduced amino acid sequences of these alleles exhibited 95.6–100.0% identity. Six of nine sequences were identical, as shown in Fig. 1, whereas three had a limited set of amino acid substitutions. In one isolate (175/96), an internal deletion of 14 residues was found, whereas in another isolate (263/96) the decapeptide NRINQTEEDS encompassing amino acid residues 210–219 of the predominant FrpD sequence was replaced by an unrelated octapeptide YHRYGEND (Fig. 1). Altogether these results suggest that the sequence of FrpD is quite conserved across serogroups of meningococci.

FrpD Is Produced under Conditions of Low Iron Availability—As illustrated in Fig. 2A, the frpD and frpC genes can be predicted to form an iron-regulated frpDC operon, transcribed from a promoter containing a binding site for the ferric uptake regulator protein Fur at −300-bp upstream the start of frpD gene (7). To examine whether production of FrpD also depends on iron availability, meningococci were grown in medium containing 100 μM desferrioxamine B to chelate free ferric ions, or in medium supplemented with 7 mM ferric nitrate as iron source, and the specific presence of iron-regulated proteins was assessed by two-dimensional gel electrophoresis (IEF/SDS-PAGE). Among the protein species present only in extracts from iron-limited cultures,2 the electrophoretic mobility of one protein matched the predicted FrpD molecular mass of 30 kDa and pl of ~5.6 (Fig. 2B). This protein was unambiguously identified as FrpD by peptide mass fingerprint mapping (64% coverage of the FrpD sequence) as documented in Table I.

FrpD Protein May Be Produced in Several Forms—Inspection of the 271-residue long FrpD sequence (FrpD271), indicated that FrpD could be produced with a somewhat atypical N-terminal signal peptide, possibly promoting FrpD export across the cytoplasmic membrane. Moreover, as illustrated in Fig. 3A, the signal peptide of FrpD271 could potentially be processed at two different sites, with a cleavage site for type I general signal peptidase predicted between residues Thr21 and Met22 and an alternative cleavage site predicted between residues Ser24 and Cys25, possibly recognized by the type II signal peptidase processing bacterial lipoproteins. As, however, already noted by Thompson et al. (7), the frpD gene sequence also contains two potential translation initiation sites located 21 codons apart, which could be giving rise to production of two differentially located forms of FrpD (Fig. 3A). Remarkably, initiation of FrpD synthesis at the second site would yield a cytosolic protein of 250 residues, lacking the signal peptide (FrpD250), but having the same N-terminal sequence as the polypeptide resulting from processing of FrpD271 at the predicted signal peptidase I cleavage site (Fig. 3A). A conclusive characterization of this possibly complex pattern of expressed FrpD forms was, however, not possible in N. meningitidis, because of the very low FrpD amounts produced (Fig. 2B and data not shown). The functionality of the FrpD signal peptide and subcellular localization of recombinant FrpD271 were, therefore, examined upon expression in E. coli.

FrpD Is Exported to the Cell Envelope—To allow straightforward detection and purification of FrpD, a C-terminal His6 affinity tag was fused to the recombinant FrpD271 and FrpD250 proteins. Upon expression of the frpD271-6xHis and frpD250-6xHis constructs in E. coli under the control of strong T7 phage transcription and translation initiation signals, however, only the truncated FrpD250-6xHis protein (~30 kDa), lacking the predicted signal peptide, was found to be overproduced in E. coli (Fig. 3B). The FrpD250-6xHis protein with the putative signal peptide was produced at much lower levels, as confirmed by immunodetection (Fig. 3B). Such result could be expected if

\[ \text{FrpD} \rightarrow \text{FrpD} \rightarrow \text{FrpD} \]
FRP D IS EXPORTED TO THE OUTER BACTERIAL MEMBRANE—To analyze the membrane localization of FRP D in more detail, it was particularly important to avoid overproduction of FRP D precursors that could be overwhelming the export machinery and cause a biased subcellular localization of FRP D. Therefore, the FRP D 271-6xHis protein was expressed from the moderate copy number pSU39 vector at low levels, and cellular envelopes containing moderate but detectable FRP D amounts were separated into the inner and outer membrane fractions by equilibrium flotation in sucrose density gradients.

As documented in Fig. 5 by a typical result of the obtained membrane separation, the lightest gradient fractions with sucrose concentrations ranging from 30 to 40% (w/w), containing inner membrane vesicles, and exhibiting the highest activity of the cytoplasmic membrane marker enzyme NADH-oxidase, contained two FRP D forms. Edman degradation performed on the larger form of FRP D, affinity-purified from the inner membrane fractions, yielded an N-terminal sequence of (M)RPYPAT corresponding to the FRP D 271 precursor with an unprocessed signal peptide. This FRP D 271 precursor accounted for about 25–30% of the total membrane-associated FRP D, judged from the semiquantitative immunoblot analysis. Most of the membrane-associated FRP D protein (∼60%) was, however, detected as a processed protein of lower molecular mass. This was partly present in inner membrane fractions and mostly floated at sucrose concentrations ranging from 44 to 50% (w/w), at which typically the outer membrane fraction of E. coli is recovered. These fractions were, indeed, also enriched for the outer membrane marker protein LamB. While identity of the FRP D protein affinity-purified from these gradient fractions could be unambiguously confirmed by MALDI-TOF MS analysis (not shown), Edman degradation failed to yield an N-terminal sequence for this FRP D protein form, suggesting that its N terminus was, indeed, blocked by lipidation (see above). Altogether, these results show that the 271-residue-long FRP D protein is produced with a functional lipoprotein signal peptide, and its mature form is sorted to the outer membrane.

FRP D BINDS WITH HIGH AFFINITY TO THE N-TERMINAL PART OF FRP C—In search for the unknown biological activity of FRP C, we expressed it together with FRP D in E. coli cells, because other accessory proteins of certain rtx determinants were shown to activate RTX toxins by post-translational modification. While no activation of FRP C by FRP D could be detected (data not shown), it was observed that both FRP D 271 and FRP D 250 co-purified with FRP C under the rather stringent conditions of chitin affinity chromatography of the FRP C chitin binding domain fusion protein (Fig. 6). This suggested a strong interaction between the two proteins, and the purified FRP D and FRP C proteins could, indeed, be cross-linked with an apparent 2:1 stoichiometry by the short-arm bifunctional cross-linking reagent DSP, which can cross-link only proteins forming stable complexes (data not shown). Therefore, a solid phase binding assay was designed to further characterize the interaction of FRP D with FRP C.

Affinity-purified FRP C (8) or FRP D 250-6xHis proteins, respectively, were allowed to bind the other FRP ligand coated on microplate wells over a range of concentrations, and the levels of FRP D-FRP C complexes formed were determined by immunodetection. As documented in Fig. 7, FRP D bound to adsorbed FRP C with a high apparent affinity, in a highly concentration-dependent and saturable manner, and the same was also true in a reverse assay setup for binding of FRP C to coated FRP D. The apparent K_d dissociation constant of the FRP D-FRP C complex, as derived following non-linear regression fitting of the obtained data, was found to be in the subnanomolar range between 0.16 and 0.24 nM (Fig. 7). Furthermore, no significant

### Table 1

| Peptide position in FRP D sequence (residues) | [M + H]^+ measured | [M + H]^+ calculated |
|---------------------------------------------|---------------------|---------------------|
| 44–69                                       | 2898.4              | 2898.3              |
| 70–79                                       | 1182.6              | 1182.6              |
| 111–126                                     | 1949.6              | 1948.0              |
| 127–134                                     | 1819.9              | 1819.9              |
| 135–146                                     | 1031.6              | 1031.6              |
| 135–147                                     | 1444.7              | 1444.8              |
| 158–162                                     | 1848.9              | 1848.8              |
| 185–197                                     | 1997.1              | 1997.0              |
| 198–208                                     | 1235.6              | 1235.7              |
| 219–208                                     | 1107.5              | 1107.6              |
| 212–240                                     | 3375.6              | 3375.5              |
| 243–270                                     | 3286.7              | 3286.6              |

a Meningococcal proteins produced under iron-limited growth conditions were separated by two-dimensional electrophoresis, the stained protein spot matching the predicted pl and M_r values of FRP D was excised from SDS-PAGE gels, subjected to in-gel tryptic digestion, and the protonated masses [M + H]^+ of the extracted peptides were determined by MALDI-TOF MS analysis.
b Masses computed for FRP D peptide fragments expected to result from tryptic digestion of N. meningitidis FRP D (Swissprot. acc. no. S35026).

FRP D IS A LIPOPROTEIN—We next examined whether FRP D 271 is processed to a lipoprotein. Production of FRP D 271 or the corresponding first 72 residues of FRP D 250, labeled protein species were detected in cells producing FRP D, or the corresponding first 72 residues of FRP D 250, respectively, were allowed to bind the other FRP ligand coated on microplate wells over a range of concentrations, and the levels of FRP D-FRP C complexes formed were determined by immunodetection. As documented in Fig. 7, FRP D bound to adsorbed FRP C with a high apparent affinity, in a highly concentration-dependent and saturable manner, and the same was also true in a reverse assay setup for binding of FRP C to coated FRP D. The apparent K_d dissociation constant of the FRP D-FRP C complex, as derived following non-linear regression fitting of the obtained data, was found to be in the subnanomolar range between 0.16 and 0.24 nM (Fig. 7). Furthermore, no significant
effect of the lipidation of FrpD, or of the presence of the un-
processed signal peptide on the affinity of the protein toward
FrpC, was observed (data not shown).

To localize the portion of the 1829-residue long multidomain
FrpC protein that was involved in the binding interaction with
FrpD, a set of intein-CBD-tagged deletion mutants of FrpC
(Fig. 8) was constructed, purified by chitin affinity chromatog-
raphy, and examined for FrpD binding. As summarized in
Table II, besides the protein lacking the entire non-repetitive
N-terminal portion of FrpC (FrpC[/H9004]1–862), two of the deletion
mutants, the FrpC[/H9004]1–199 and FrpC[/H9004]108–300 constructs, har-
boring deletions within the first 300 amino acid residues of
FrpC, failed to bind FrpD. The construct with deletion of resi-
dues 200–397 also exhibited an importantly reduced affinity
(Kd/H11011200 nM). In contrast, the proteins with deletions
of adjacent portions beyond residue 400 (FrpC[/H9004]400–448 and
FrpC[/H9004]451–861) were much less affected in binding to FrpD
(Kd/H110114n M and Kd/H1101110 nM, respectively), suggesting that the FrpD binding
site was localized within the first 300 residues of FrpC.

FrpD Binding Does Not Affect the Calcium-induced Autocat-
alytic Processing of FrpC—We have recently shown that in the
presence of calcium ions the FrpC protein undergoes an auto-
neisseria lipoprotein frpD binds RTX protein frpC
FIG. 3. FrpD has a functional N-terminal secretion signal. A, location of the two possible ribosome binding sites (RBS) and translation
initiation sites found in frpD that might allow production of two different protein products of FrpD. The 271-amino acid residue long FrpD[/H9004]271
precursor would be produced with a signal peptide containing predicted cleavage sites for both type I and type II signal peptidases (indicated by
arrows). The putative 250-residue long cytosolic protein FrpD[/H9004]250 would be devoid of a signal peptide. B, expression of FrpD in E. coli. Recombinant
FrpD[/H9004]271 and FrpD[/H9004]250 proteins were expressed in E. coli BL21(ADE3), crude cell extracts were separated by SDS-PAGE (10%) and stained with
Coomassie Blue, or transferred to a polyvinylidene difluoride membrane and immunodetected using an antibody recognizing the C-terminal His 6
tag of both the FrpD[/H9004]271 and FrpD[/H9004]250 proteins, respectively. An extract of mock E. coli BL21(ADE3) was used as a negative control. C, schematic
representation of the FrpD-PhoA fusions generated by fusing the first 93-amino acid residues of FrpD[/H9004]271, or the first 72 residues of FrpD[/H9004]250 to a
signal-less PhoA moiety, respectively. D, phenotypes of E. coli XL1-Blue cells expressing the FrpD[1–93]-PhoA or FrpD[22–93]-PhoA fusions, the
FrpD[/H9004]271 protein alone, and the PhoA moiety alone, respectively. Bacteria were grown on Luria-Bertani agar plates containing the chromogenic
PhoA substrate BCIP. Alkaline phosphatase activity was detected as a blue-green (dark) phenotype of colonies.
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FIG. 4. FrpD is a lipoprotein. E. coli BL21(ΔDE3) transformants harboring the pT7-7 vector or the derived constructs for expression of the FrpD271 and FrpD250 proteins, respectively, were grown to mid-exponential phase in M9 medium supplemented with 0.4% glucose, induced by 1 mM IPTG, and membrane lipid moieties of the expressed lipoproteins were labeled upon addition of [9,10(n)-3H]palmitic acid for 3 h. Cell lysates were separated by SDS-PAGE (10%), and the dried gel was analyzed by autoradiography.

FIG. 5. FrpD is exported to the outer bacterial membrane. Bacterial membranes were prepared from IPTG-induced cells of E. coli BL21 carrying pSU39/frpD271 as described under “Experimental Procedures” and subjected to fractionation on sucrose density gradients (30–62% (w/w) sucrose) by flotation to equilibrium in 40 h at 10 °C. Gradient fractions were analyzed for the presence of FrpD by immunoblotting with anti-polyhistidine monoclonal antibody. Amounts of NADH oxidase activity were determined for the cytoplasmic membrane marker, and the presence of the LamB protein was monitored for the outer membrane marker.

FIG. 6. FrpD co-purifies with FrpC. A, schematic representation of the reconstructed frpD-frpC operon used for co-expression of FrpD271, or of FrpD250, together with FrpC in E. coli. The frpD and frpC open reading frames were placed under the control of an IPTG-inducible T7 promoter (pT7) and fused to translation initiation signals of gene 10 of bacteriophage T7 (black diamonds). The frpC open reading frame was next fused in-frame to a C-terminal intein (int) chitin binding domain (cbd) self-excisable tag, which allowed affinity purification of FrpC on chitin beads as previously described (8). B, copurification of FrpD250 with FrpC-intein-CBD on chitin beads. FrpC-intein-CBD was purified on chitin beads columns from the cytosolic fraction of E. coli BL21(ΔDE3) carrying pTYB2-frpD250-frpC. Purification steps were analyzed using a 7.5–12% gradient SDS-PAGE gel and in parallel to Coomassie Blue staining (Total protein), the purified FrpC and copurified FrpD proteins were immunodetected in the eluted fractions using specific polyclonal antisera (Immunodetection). The loaded fractions were: CL, clarified E. coli lysate; FT, chitin column flow through; W, column wash; E, eluted FrpC fraction.

Many meningococcal proteins expressed at low iron conditions are exported into or across the cell envelope to mediate iron uptake or host colonization (46–48). We show here that the iron-regulated protein FrpD, lacking homology to any known sequences in public databases, is a lipoprotein localized to the bacterial outer membrane. FrpD binds with high affinity to the first 300-residue portion of the RTX protein FrpC, which strongly suggests that FrpD is a new type of accessory protein of rtx genetic determinants. The biological role of FrpD in meningococcal infections is, hence, most likely linked to the thus far unknown biological activity of the RTX protein FrpC. Interestingly, however, as verified both by immunoblotting and MALDI-TOF mass spectrometric identification (not shown), the processed N-terminal FrpC fragment formed a very stable and possibly also covalently cross-linked complex with FrpD250 (Fig. 9), which could not be dissociated under the harsh denaturing conditions of SDS-PAGE sample preparation and separation.

DISCUSSION

catalytic processing between residues Asp114 and Pro115, which is followed by formation of high molecular weight (HMW) FrpC forms that are covalently cross-linked by an isopeptide bond forming between the C-terminal carboxyl group of the liberated Asp114 and an ε-amino group of a nearby lysine residue (9). It was, hence, important to examine whether FrpD binding would affect the autoprocessing activity of FrpC. As, however, documented in Fig. 9, no effect of the presence of FrpD on calcium-induced processing of FrpC was observed, either when the processed and lipidated FrpD271, or the FrpD250 proteins were incubated with FrpC at 2 mM concentrations of calcium ions for 1 h (Fig. 9) or when the kinetics of FrpC autoprocessing in the presence of FrpD was analyzed (not shown). Binding of FrpD to FrpC and the calcium-dependent autoprocessing of FrpC appear, hence, to be two independent and unrelated activities of FrpC. Interestingly, however, as verified both by immunoblotting and MALDI-TOF mass spectrometric identification (not shown), the processed N-terminal FrpC fragment formed a very stable and possibly also covalently cross-linked complex with FrpD250 (Fig. 9), which could not be dissociated under the harsh denaturing conditions of SDS-PAGE sample preparation and separation.
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FIG. 7. FrpD strongly binds FrpC in a concentration-dependent and saturable manner. A, increasing concentrations of purified FrpD250 protein were added to ELISA plate wells coated with constant amounts of purified recombinant FrpC (20 μg/ml), and complex formation was allowed to proceed for 1 h at 37 °C. Upon washing out unbound FrpD250, the amounts of formed FrpC-FrpD complex were determined by colorimetric immunodetection using rabbit polyclonal serum against FrpC and anti-rabbit IgG-peroxidase conjugate. B, plate wells were coated with constant amounts of FrpD250 (20 μg/ml), and increasing concentrations of FrpC were added to plate wells. Following incubation at 37 °C for 1 h and plate washing, the amounts of formed FrpC-FrpD complex were determined by rabbit polyclonal serum against FrpC and anti-rabbit IgG-peroxidase conjugate. The curves represent the best fit of obtained data to a sigmoidal dose-response curve obtained by nonlinear regression analysis of mean ± S.D. values from four independent determinations (n = 4). The apparent \( K_d \) values were determined as FrpD or FrpC concentrations giving a half-maximal \( A_{492} \) value for the fitted binding curve.

![FIG. 7](image)

FIG. 8. A schematic representation of the truncated FrpC protein variants. The portions deleted in the truncated FrpC protein variants are indicated by a dashed line. The numbers following the symbol Δ indicate the first and the last residue numbers of the respective portion deleted in the given protein construct.

![FIG. 8](image)

**TABLE II**

| Protein          | Apparent dissociation constant (\( K_d \)) |
|------------------|------------------------------------------|
| FrpC             | 0.20 ± 0.04                              |
| FrpCΔ(863–1829)  | 0.18 ± 0.05                              |
| FrpCΔ(1–862)     | ND\(^a\)                                 |
| FrpCΔ(1–199)     | ND\(^a\)                                 |
| FrpCΔ(108–300)   | ND\(^a\)                                 |
| FrpCΔ(200–397)   | ~200\(^d\)                               |
| FrpCΔ(400–448)   | ~4\(^d\)                                 |
| FrpCΔ(451–861)   | ~10\(^d\)                                |

\(^a\) The apparent \( K_d \) values were determined as FrpD concentrations yielding half-maximal binding, as determined from curves representing the best fit of mean ± S.D. FrpD binding values from four independent determinations (n = 4) to a sigmoidal dose-response binding curve are shown.

\(^b\) No binding of FrpD to the RTX moiety of FrpC was detected.

\(^d\) The \( K_d \) values of these mutants were difficult to determine because of the high variance of obtained values, most likely due to conformational effects of the deletions on the structure of the FrpD binding site of FrpC.

![TABLE II](image)

that FrpD plays, together with FrpC, some role in meningococcal lifestyle in colonization of humans. This would be suggested by conservation of the FrpD sequence across meningo-

![FIG. 9](image)

FIG. 9. FrpD does not inhibit the calcium-dependent autoprocessing of FrpC. Affinity-purified FrpC was incubated for 1 h at 37 °C and 2 mM free Ca\(^{2+}\) ion concentration in the presence (+) or absence (−) of purified FrpD271 or FrpD250 proteins, respectively, at a final FrpC:FrpD molar ratio of 1:2. The protein samples were separated by SDS-PAGE (7.5%) and stained with Coomassie Blue. HMW forms, high molecular weight forms of FrpC; FrpC-Cter, C-terminal fragment of FrpC; FrpC-Nter, N-terminal fragment of FrpC resulting from calcium-induced processing of the peptide bond between residues Asp\(^{411}\) and Pro\(^{415}\) of FrpC (9); FrpD, FrpD250 or FrpD271, respectively.

![FIG. 9](image)
Neisseria Lipoprotein FrpD Binds RTX Protein FrpC

...from a high copy number plasmid pT7-7 frpD protein PulS involved in secretion of genesis and/or activity by chaperoning or mediating FrpC to detect any accumulation of the cytoplasmic FrpD250 form in possibly remaining in the cytoplasm. We were, however, unable one being a lipoprotein exported to the outer membrane and one similar to that of FrpC (9).

very high affinity. This raises intriguing questions on what could form, both were able to bind the N-terminal portion of FrpC with extent in meningococci. As shown here the exported lipidated FrpD, as well as its recombinant non-exported FrpD250 protein form, both were able to bind the N-terminal portion of FrpC with very high affinity. This raises intriguing questions on what could be the biological role of such a strong binding interaction. The cytosolic and/or exported FrpD forms could, for example, be serving as inhibitors of FrpC activity in bacterial cytoplasm and/or cell envelope, safeguarding the producing cell from FrpC activity in the case of inefficient secretion and/or mislocalization. A precedent for genes encoding accessory inhibitor proteins encoded by rtX loci is found within loci for production of the secreted RTX proteases of Photorhabdus, Erwinia edent for genes encoding accessory inhibitor proteins encoded by...
The *Neisseria meningitidis* Outer Membrane Lipoprotein FrpD Binds the RTX Protein FrpC
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