Sequence variability of FrpB, a major iron-regulated outer-membrane protein in the pathogenic neisseriae

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The FrpB protein from pathogenic neisseriae is a 77 kDa iron-regulated outer-membrane protein that belongs to the family of TonB-dependent receptors and may have potential as a vaccine component. Comparison between the *frpB* gene from three different meningococcal strains and a published gonococcal one revealed that the region from residues 350 to 390 displays pronounced sequence variability. In a model for the topology of FrpB in the outer membrane, this region corresponds to loop 7, the longest of the predicted 13 surface-exposed loops. Binding of four out of a total of eight bactericidal monoclonal antibodies to synthetic peptides corresponding to loop 7 showed that their epitopes are located here. The *frpB* genes from five additional meningococcal strains were cloned and sequenced in this region. Pairwise comparisons showed different degrees of similarity.

Keywords: *Neisseria meningitidis*, iron limitation, antigenic variation, outer-membrane vaccine

INTRODUCTION

Disease caused by *Neisseria meningitidis* remains a significant public health problem worldwide. At present, there is no vaccine available against group B organisms, which are the predominant cause of meningococcal disease. The use of the group B capsular polysaccharide as a vaccine has been hindered by the presence of identical structures on neural cell adhesion molecules in the human host, raising the possibility of inducing autoimmune pathology with such a vaccine. As an alternative, vaccines based on meningococcal outer membranes are currently under investigation (Diaz Romero & Outschoorn, 1994; Poolman, 1995; Frasch, 1995). Efficacy trials in humans with such vaccines have demonstrated partial protection against meningococcal disease. They consist of outer-membrane vesicles prepared from one particular strain; since many of the major outer-membrane proteins (OMPs) are variable among different strains, a large proportion of the protective antibodies induced will be

strain-specific. In addition, not all outer-membrane components contribute to the induction of bactericidal antibodies. For these reasons, we are attempting to improve outer-membrane vesicle vaccines, by manipulating the OMP composition of a particular strain (van der Ley & Poolman, 1992; van der Ley *et al.*, 1995). Since the class 1 protein appears to be an important target for bactericidal antibodies, derivatives of strain H44/76 carrying three copies of the *porA* gene have been constructed, with each copy encoding an antigenically different form of the class 1 OMP. Expression of the class 3 and 4 OMPs was abolished because these proteins do not contribute much to the induction of bactericidal antibodies and, in the case of class 4 OMP, might even be detrimental due to the induction of blocking antibodies. Other candidates for inclusion are the several OMPs induced by growth under iron limitation, including receptors for such iron sources as lactoferrin, transferrin and haemoglobin (Legrain *et al.*, 1993; Pettersson *et al.*, 1994; Stojiljkovic *et al.*, 1995). In particular, the FrpB protein appears to be a good target for bactericidal antibodies (Pettersson *et al.*, 1990). The sequence of this 77 kDa protein shows the characteristics of a TonB-dependent outer-membrane receptor; however, no role in iron uptake has yet been demonstrated (Pettersson *et al.*, 1995). Since bactericidal monoclonal antibodies (mAbs)

Abbreviation: OMP, outer-membrane protein.

The GenBank accession numbers for the sequences determined in this work are: U55377 (2996), U55378 (892257), U67312 (870227), U67310 (870446), U67314 (M982), U67311 (M990) and U67313 (S3446).

isolated against FrpB were found to be strain specific, characterization of the variability of this protein is essential for assessment of its vaccine potential. This study reports the sequence variability of the *frpB* gene among a number of different strains, and the mapping of epitopes recognized by bactericidal mAbs.

METHODS

Bacterial strains. The *Neisseria meningitidis* group B strains H44/76 (15:P1.7,16), 2996 (2b:P1.5,2), 892257 (4:P1.7^h,4), 870227 (4:P1.5^h,10), 870446 (14:P1.12,13), M982 (9:P1.9), M990 (6:P1.6) and S3446 (14:P1.14) were from our own collection and have been described previously (van der Ley *et al.*, 1995). Meningococci were grown overnight at 37 °C on GC medium base (Difco) supplemented with IsoVitaleX in a humid atmosphere containing 5% CO₂. *Escherichia coli* strain INVaF' was grown on LB medium at 37 °C.

Sequence analysis. PCR reactions on meningococcal genomic DNA were performed with AmpliTaq on whole-cell suspensions that had been heated for 10 min at 95 °C. Primers 4 (5'-GGCTGCAGAGGGTATTGGTCCAGCGTTG-3') and 12 (5'-GCAGAAAATAATGCCAAGGTC-3'), based on the *frpB* sequence previously published for strain H44/76, were used in the following programme: 30 cycles of 1 min at 95 °C, 1.5 min at 52 °C, 2 min at 72 °C. The resulting PCR products of approximately 2.0 kb were cloned into the plasmid vector pCRII using the TA cloning kit of Invitrogen. Plasmid DNA was purified after alkaline lysis on Qiagen columns. Sequence analysis was performed with an Applied Biosystems automatic sequencer on double-stranded plasmid DNA templates, with the Taq Dye Deoxy Terminator Cycle Sequencing Kit and *frpB*-specific primers. Sequences were established for both strands and with at least two independent clones.

Peptide ELISA. Biotinylaminoheptyl peptides were synthesized using an automated multiple peptide synthesizer (Wiertz *et al.*, 1992). They were bound to avidin-coated microtitre plates and used in an ELISA as described by Pettersson *et al.* (1994). The bactericidal mAbs directed against FrpB from strain H44/76 (mn70K1, mn70K2, mn70K3, mn70K5, mn70K6 and mn70K7) and strain 2996 (A1A-A2 and 4F3-E5) have been described previously (Pettersson *et al.*, 1990, 1991).

RESULTS AND DISCUSSION

Sequence of the *frpB* gene in different meningococcal strains

In a previous study, FrpB-specific mAbs with bactericidal activity against either strain H44/76 or strain 2996 were described. Most strains from a collection of 74 failed to react with any of these antibodies, suggesting a high degree of antigenic heterogeneity in this protein (Pettersson *et al.*, 1990). The *frpB* gene from strain H44/76 has been sequenced previously (Pettersson *et al.*, 1995); we now report the complete sequences for strains 2996 and 892257. Since the latter strain is not recognized by any of the isolated mAbs, its FrpB protein must represent a third antigenic type. In Fig. 1, a comparison is given of the resulting three meningococcal sequences with the FrpB sequence that was reported for the gonococcal strain FA19 (Beucher & Sparling, 1995). The overall homology varies from 91 to 94%, with the most pronounced

Reference molecule:	FA19	1 - 691	(691 aa)	Homology
Sequence 2:	892257	1 - 692	(692 aa)	94%
Sequence 3:	2996	1 - 697	(697 aa)	91%
Sequence 4:	H44/76	1 - 698	(698 aa)	92%

AENNANVALDTVTVKGDROGSKIRTNIVTLQKKDESTATDMRELLKEEPSIDFGGGNGTSQFLTLRGMGQ	
.....K.V.....	
.....K.....	
.....K.V.....	
NSVDIKVDNAYSDSQILYHQGRFIVDPALVKVSVQKGAGSASAGIGATNGAITAKTVDAQDLKGLDKN	
.....V.....G.....	
WGVRNLNSGFAGNNGASVGSVFGKEGNFDGLFSYNRDEKDYEAGKGFND-NGGKTVTPYSALDKRSYLA	
.....PSDS.E.V.....N.....NF.....	
.....S.E.V.....V.T.....	
.....S.E.V.....V.....	
KIGTTFGDGHRIVLHSMKQDHRGIRTVREFAVSEKNSRITIKRQAPSYRETTQSNLNTAYTGKDLGFV	
.....T.GA.D.....N.....V.A.....N.....	
.....S.GD.....G.....T.TN.....LDLD.....A.....	
.....D.....T.GD.S.....N.D.....A.....N.....	
EKLDANAYVLEKKRVSADDDKNGYAGNVKGNHTRATRSNPNFDSRLAEQTLKYGINYRHQEIKPQA	
.....E.....SGT.....T.GA.....	
.....E.....T.G.....	
.....E.....SGT.....T.GA.....	
FLNSEFEIKDKEKATNEKK-----KN-----RENEKIAKAYRLTNPTKTDGAYIEAHE	
.....Q.K.E.....D.....N.....A.....	
.....K.S.PTT.....KNGQDVA.....PADQQAKD.KD.ALVHS.....	
.....K.S.....P.T.....NGQKVDKPMQQM.D.....AD.DTVH.....K.S.....V.V.....D	
IDGFTLTGLRYDRFKVKTGDKTVSSSLNPSFGVIWQPREHWSFSASHNYAGRSPLRYDALQTHGKRG	
.....N.....H.....S.....	
.....N.....N.....H.....S.....	
.....GD.....N.....H.....S.....	
IISTADGTKAERARNTIEGFNYNDGTFAANGSYFRQTKDALANPQRNHRSDVAVREAVNAGYIKNHGYEL	
.....G.....E.....R.W.....V.A.....	
.....W.....	
GASYRTGGLTAKVGVSHSKPRFYDTHDKLLSANPEFGAQQVGRWTASLAYRFKNPNLEIGWRGRYVQKA	
.....Q.....	
.....PK.....Q.....Q.....	
.....Q.....	
VGSILAAGQKDRDGLKLVNVRQGFVNDVFNWKLPGKDTLVNVLNVNVPDKFYFPHSRQWNTLPGVG	
.....V.....N.....K.....I.....NT.....D.....	
.....V.....N.....K.....H.....NT.....D.....	
.....T.....K.N.....K.....N.....	
RDVRLGVNXYF	
.....	
.....	

Fig. 1. Sequence comparison of the mature FrpB protein from *Neisseria gonorrhoeae* strain FA19 (Beucher & Sparling, 1995) and *Neisseria meningitidis* strains 892257, 2996 and H44/76 (Pettersson *et al.*, 1995; this study).

sequence variation confined to the region from residues 350 to 390. In a model for the topology of FrpB in the outer membrane, 13 surface-exposed loops were predicted (Pettersson *et al.*, 1995). The longest of these, loop 7, corresponds to this region of highest sequence variation (Fig. 2). Most of the other, shorter, polymorphic regions are also located in surface-exposed loops of the model; particularly great variability is also found within the predicted loop 5, the second-largest loop. An exception is the variable region around residue 150, which is predicted to be at the periplasmic side. Remarkably, the loop 7 sequence from strain 892257 is highly similar to the gonococcal sequence but very different from those of the two other meningococcal strains. However, in the rest of the sequence the similarity between the meningococcal strain 892257 and the gonococcal strain FA19 is not particularly close. This situation is reminiscent of the *porA* gene, where a mosaic structure has been interpreted as evidence for horizontal gene transfer among different meningococcal strains by means of genetic transformation (Feavers *et al.*, 1992). Interspecies transfer of chromosomal genes between *N. meningitidis* and *N. gonorrhoeae* has been suggested for the *thpA/B* and *porB* genes

Fig. 2. Topology of FrpB from strain H44/76 in the outer membrane (taken from Pettersson *et al.*, 1995). The most variable regions are boxed. The minimal sequence essential for binding of bactericidal mAbs is shaded.

(Cornelissen & Sparling, 1994; Vázquez *et al.* 1995), encoding the transferrin receptor and the major porin, respectively. In both cases, meningococcal sequences were found that are much closer to a gonococcal sequence than to other meningococcal strains. In the case of *frpB*, a relatively recent exchange of the loop 7 region between ancestors of gonococcal strain FA19 and meningococcal strain 892257 is likely.

Location of epitopes recognized by FrpB-specific mAbs

Since loop 7 in the FrpB topology model is both the longest surface-exposed loop and the region with the most pronounced sequence variation, it seemed likely that this would also constitute an important target for antibodies. Previously, six bactericidal mAbs specific for FrpB from strain H44/76, and two against strain 2996, have been described (Pettersson *et al.*, 1990, 1991). In order to map their epitopes, their binding to synthetic peptides derived from the loop 7 sequence was investigated. Four 18-residue peptides with a 12-residue overlap were synthesized from both the H44/76 and the 2996 sequences (Fig. 3), and mAb binding was measured by ELISA (Fig. 4, Table 1). Two of the four H44/76 peptides were recognized by three mAbs, the first peptide giving the strongest reactivity. In the case of the four 2996 peptides, only the first one was recognized by one mAb. The first peptides correspond to the top of loop 7, showing that this is indeed a likely epitope for bactericidal

H44/76 loop 7 peptides:

KNGQKVDKPMQQMKDRA	124E2
DKPMQQMKDRAEDTVH	124A3
QMKDRAEDTVHAYKLSN	124A2
DEDTVHAYKLSNPTKTDT	124C2

2996 loop 7 peptides:

KNGQDVAKPADQQAQDRK	124D2
AKPADQQAQDRKDEALVH	124F2
QAQDRKDEALVHSYRLTN	124F1
DEALVHSYRLTNPTKTDT	124B2

Fig. 3. Synthetic overlapping peptides based on the loop 7 sequence of FrpB from strains H44/76 and 2996.

mAbs. The remaining mAbs might be specific for another loop in FrpB, or for a particular sequence and/or conformation in loop 7 that is not represented by the four peptides. However, it should be noted that all these mAbs can bind to denatured FrpB in a Western blot.

Sequence variation in the loop 7 region of FrpB

As described above, loop 7 in the FrpB topology model contains both a highly variable part of the protein and the epitopes for a number of bactericidal mAbs. In order to gain more insight into the degree of interstrain variation

Fig. 4. Binding of FrpB-specific mAbs mn70K1 and 4F3-E5 to synthetic peptides derived from the loop 7 sequence of strain H44/76 and 2996. ELISA reactivity is expressed as A_{450} . (a, b) mn70K1 against H44/76 peptides (a) and 2996 peptides (b); (c, d) 4F3-E5 against H44/76 peptides (c) and 2996 peptides (d). Two other mAbs (mn70K2 and mn70K3) gave results similar to mn70K1; the remaining four (three against H44/76 and one against 2996) failed to react with any of the peptides.

Table 1. Binding of FrpB-specific mAbs to synthetic peptides corresponding to loop 7

The results are based on ELISA reactivity as shown in Fig. 4.

mAb	H44/76 peptides				2996 peptides			
	124E2	124A3	124A2	124C2	124D2	124F2	124F1	124B2
mn70K1	++	+	—	—	—	—	—	—
mn70K2	++	+	—	—	—	—	—	—
mn70K3	++	+	—	—	—	—	—	—
mn70K5	—	—	—	—	—	—	—	—
mn70K6	—	—	—	—	—	—	—	—
mn70K7	—	—	—	—	—	—	—	—
A1A-A2	—	—	—	—	—	—	—	—
4F3-E5	—	—	—	—	++	—	—	—

within this immunologically important part of FrpB, its sequence was determined for five other meningococcal strains, each representing a different porin serotype/subtype. A region of approximately 100 amino acid residues around loop 7 is shown for all eight strains in Fig. 5. Pairwise comparisons show variable levels of similarity (Table 2). Two sequences are identical (2996 and 870446) and two others are nearly so (870227 and S3446). However, even when a relatively high degree of similarity is found, the proteins can still be antigenically different, as is the case for strains H44/76 and 2996. In this set of strains, there is no correlation between serotype/subtype on the one hand, and FrpB loop 7 sequence on the other. For instance, both strains 870446 and S3446 are serotype 14, but have highly different loop 7 sequences. The identical loop 7 sequences in strains 2996 and 870446 are found in combination with completely different serotype/subtype, i.e. 2b:P1.5,2 and 14:P1.12,13 respectively. This

again indicates horizontal transfer of *porA/B* and/or *frpB* alleles among different strains.

Conclusions

In this study, sequence analysis and epitope mapping of the meningococcal FrpB protein have been used to demonstrate that the longest surface-exposed loop in the previously proposed topology model is both hyper-variable and a target for bactericidal mAbs. A similar correlation has also been found for the neisserial porins (van der Ley *et al.*, 1991). In the case of the gonococcal PIA porin, statistical analysis of synonymous and non-synonymous substitutions has been used to infer positive Darwinian selection for diversity in the surface-exposed loops, driven by the human immune system (Smith *et al.*, 1995). Considering the very high variability of loop 7 of FrpB, it is tempting to speculate that this not only has the

Reference molecule:	H44/76	1 - 105	(105 aa)	Homology
Sequence 2:	2996	1 - 105	(105 aa)	83%
Sequence 3:	870446	1 - 105	(105 aa)	83%
Sequence 4:	892257	1 - 98	(98 aa)	70%
Sequence 5:	M990	1 - 98	(98 aa)	76%
Sequence 6:	870227	1 - 108	(108 aa)	73%
Sequence 7:	S3446	1 - 108	(108 aa)	73%
Sequence 8:	M982	1 - 108	(108 aa)	70%

H44/76	PNHTRITTRGANFNFDRLAEQTLKYGINYRHEIKPQAFNSKFSIP-	(49)
2996	M.....	(49)
870446	M.....	(49)
892257	Q.K.ED	(50)
M990	M.....	(48)
870227	M.....GE----	(46)
S3446	M.....GE----	(46)
M982	M.....GE.K.S-	(49)
H44/76	---TTEEKNGQKV---D-----KPMEQQMKDRAEDTVHAYKLSN	(83)
2996	-----K....D---A-----AD..A...K..AL..S.R.T.	(83)
870446	-----K....D---A-----AD..A...K..AL..S.R.T.	(83)
892257	KEKA.D.....N.EN.KIAK..R.T.	(76)
M990	-----D.T....A-----T.A.I..N...A.A.D....	(76)
870227	-----IS.K.K---PKDPRKEID.TD.EKA..KK.M.L..S....	(86)
S3446	-----IS.K.K---PKDPRKEID.TA.EKA..KK.M.L..S....	(86)
M982	----DKKPNPANPKEEI-----DAAEKE..KK.MAL..S....	(86)
H44/76	PTKTDGTVYVEAIHDIIGDFTLT	(105)
2996	A.I....E.NG....	(105)
870446	A.I....E.NG....	(105)
892257	A.A.I....E.DG....	(98)
M990	A.I.T..E..G....	(98)
870227	A.I.T..E..G....	(108)
S3446	A.I.T..E.DG....	(108)
M982	.A.....A.I.T.....	(108)

Fig. 5. Sequence comparison of the loop 7 region of FrpB from eight different meningococcal strains. The H44/76 sequence is used as the reference.

Table 2. Similarity matrix of the FrpB loop 7 region from eight different strains

The results show percentage homology for the sequences in Fig. 5, allowing for conservative substitutions.

	H44/76	2996	870446	892257	M990	870227	S3446	M982
H44/76		90	90	80	80	81	82	84
2996	90		100	80	82	86	85	88
870446	90	100		80	82	86	85	88
892257	85	85	85		87	87	89	87
M990	86	88	88	88		84	82	85
870227	78	82	82	79	76		97	89
S3446	79	82	82	81	74	97		88
M982	81	85	85	79	78	89	89	

same basis, but might even be the function of this part of the protein, i.e. a highly exposed immune 'decoy' to protect other more conserved and functionally important surface-exposed loops. Although a function for FrpB in iron uptake has not yet been demonstrated, its homology to other TonB-dependent outer-membrane receptors strongly suggests that it must function as a receptor for some iron source or other compound that is required under conditions of iron limitation. It is unlikely that such a highly variable sequence as loop 7 could be directly involved in this presumed receptor function. The fact that in our limited set of eight strains there is already a high degree of FrpB sequence variation makes it unlikely that a vaccine based on the variable part of this protein could

ever be effective. This contradicts earlier studies suggesting that FrpB is antigenically conserved (Black *et al.*, 1986; Banerjee-Bhatnagar & Frasch, 1990). As an alternative, one could study the immunogenicity of mutant versions of FrpB lacking part of loop 7, to see if that makes it possible to induce bactericidal antibodies against other, more conserved loops.

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