Characterization of *Neisseria meningitidis* Isolates That Do Not Express the Virulence Factor and Vaccine Antigen Factor H Binding Protein

Jay Lucidarme, Lionel Tan, Rachel M. Exley, Jamie Findlow, Ray Borrow, and Christoph M. Tang

*Vaccine Evaluation Unit, Health Protection Agency, North West Regional Laboratory, P.O. Box 209, Clinical Sciences Building II, Manchester Royal Infirmary, Manchester M13 9WZ, United Kingdom*; *Centre for Molecular Microbiology and Infection, Department of Microbiology, Flowers Building, Imperial College London, London SW7 2AZ, United Kingdom*; *and University of Manchester, Inflammation Sciences Research Group, School of Translational Medicine, Stopford Building, Manchester M13 9PL, United Kingdom*

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*Neisseria meningitidis* remains a leading cause of bacterial sepsis and meningitis. Complement is a key component of natural immunity against this important human pathogen, which has evolved multiple mechanisms to evade complement-mediated lysis. One approach adopted by the meningococcus is to recruit a human negative regulator of the complement system, factor H (fH), to its surface via a lipoprotein, factor H binding protein (fHbp). Additionally, fHbp is a key antigen in vaccines currently being evaluated in clinical trials. Here we characterize strains of *N. meningitidis* from several distinct clonal complexes which do not express fHbp; all strains were recovered from patients with disseminated meningococcal disease. We demonstrate that these strains have either a frameshift mutation in the *fHbp* open reading frame or have entirely lost *fHbp* and some flanking sequences. No fH binding was detected to other ligands among the *fHbp*-negative strains. The implications of these findings for meningococcal pathogenesis and prevention are discussed.

*Neisseria meningitidis* is a Gram-negative bacterium that is a frequent member of the human nasopharyngeal flora, where it causes asymptomatic infection in 10 to 40% of healthy individuals (7, 51). Occasionally, the bacterium translocates across the respiratory epithelial barrier, via a transcellular route (45), and establishes disseminated disease by invading into and replicating within the intravascular compartment. From there, the meningococcus can spread to the cerebrospinal fluid, causing meningitis (20, 48). The organism remains a leading cause of Gram-negative septic shock and meningitis in developing countries and is responsible for epidemics that can involve hundreds of thousands of children and young adults in Saharan Africa each year (20).

The prognosis of meningococcal disease is directly correlated with levels of circulating lipooligosaccharide (LOS) and bacteremia, which can reach up to 10⁹ CFU/ml in individuals with septic shock (5), a condition which still carries a significant case fatality rate and causes substantial long-term disabilities in survivors (44). To attain such high levels within the circulation, the bacterium must avoid killing by the host immune system (39). Complement is essential for defense against meningococcal infection. This is evident from the observation that individuals with deficiency in components of the membrane attack complex (MAC), a pore-forming multiprotein complex that causes bacterial lysis, are highly susceptible to meningococcal sepsis, with over a thousandfold-increased lifetime risk of developing disease (11). Furthermore, polymorphisms or deficits of other complement factors, including C2, C3, and properdin (11), are also associated with increased risk of developing meningococcal disease, while a recent genome-wide association study demonstrated that a region on chromosome 1 harboring the gene encoding factor H (fH), the main negative regulator of the complement system, is linked to susceptibility to meningococcal disease (10).

The meningococcus has evolved multiple mechanisms that promote resistance against complement-mediated lysis. Virtually all invasive isolates recovered from individuals with meningococcal disease express a capsular polysaccharide (17), which is necessary for survival in human serum, while truncation of LOS greatly increases sensitivity to complement (16). More recently it has been shown that the meningococcus recruits fH to its surface (28, 39), which downregulates the activity of the alternative complement pathway and increases bacterial survival in the presence of human serum. fH is composed of 20 short consensus repeats (SCRs), each consisting of approximately 60 amino acids, which can engage other complement factors, including C3b, to mediate the regulatory functions of this protein (52). fH is present in the serum and binds to the surface of endothelial cells via polyanions, such as glucosaminy glycans. The meningococcus recruits fH to its surface by expressing factor H binding protein (fHbp) (28), a 27-kDa lipoprotein that consists of two β-barrels joined by a short amino acid linker (31, 40). While charged carbohydrates on the surface of the vascular endothelium engage fH, charged amino acids in fHbp bind fH at nanomolar affinities at the same site.
of this complement regulator (40). In addition, it has been shown that fH can also bind to NspA on the surface of some meningococcal strains (24).

Based on differences in the nucleotide and predicted amino acid sequences, fHbps from different strains have been categorized using multiple schemes. These include two subfamilies (A and B) (33) or three variant groups (V1, V2, and V3) (32), and recombinant antigens, including a chimeric protein consisting of a V1 fHbp fused to another protein (14).

The vaccine being tested by Pfizer consists of two fHbp protein subvariants (one V1 and one V3) (13), while the Novartis vaccine contains outer membrane vesicle (OMV) and recombinant antigens, including a chimeric protein consisting of a V1 fHbp fused to another protein (14).

During a recent genotypic analysis of potential vaccine antigens, we identified strains from individuals with invasive meningococcal disease in which fHbp was either contained a frameshift mutation or was entirely absent; these isolates were likely to be deficient in expression of this important virulence factor (40). In addition, immunization of mice with fHbps from variants 2 and 3 generates responses with some degree of immunological cross-reactivity, but these variants do not induce bactericidal antibodies against strains expressing V1 fHbp (32). The vaccine being tested by Pfizer consists of two fHbp protein subvariants (one V1 and one V3) (13), while the Novartis vaccine contains outer membrane vesicle (OMV) and recombinant antigens, including a chimeric protein consisting of a V1 fHbp fused to another protein (14).

TABLE 1. Strains used in this study

| Yr isolated | Isolate | Group | ST | cc | fHbp | Site | Presentation, age |
|-------------|---------|-------|----|----|------|------|-------------------|
| 1998        | M08 253564 | C     | 11 | T366 | 1    | 82   | 78                |
| 2000        | M00 242764 | C     | 11 | T366 | 1    | 82   | 78                |
| 2000        | M00 242809 | C     | 11 | T366 | 1    | 82   | 78                |
| 2005        | M05 240072 | B     | 11 | T366 | 1    | No ID | No ID             |
| 2006        | M06 241270 | B     | 11 | T366 | 1    | 82   | 78                |
| 2007        | M07 241093 | C     | 11 | T366 | 1    | 82   | 78                |
| 2008        | M08 240254 | B     | 11 | T366 | 1    | 82   | 78                |
| 2008        | M08 240409 | C     | 11 | T366 | 1    | 82   | 78                |
| 2008        | M08 240270 | C     | 7664 | T366 | 1    | 82   | 78                |
| 2001        | M01 240074 | C     | 11 | T366 | 1    | No ID | No ID             |
| 2008        | M08 240219 | B     | 162 | 162 | 8650 | 2    | 21   | 21                |
| 2008        | M08 240039 | B     | 162 | 162 | 8650 | 2    | 21   | 21                |
| 2008        | M08 240374 | B     | 162 | 162 | 78   | 2    | 21   | 21                |
| 2007        | M07 240804 | B     | 162 | 162 | 78   | 2    | 21   | 21                |
| 1999        | M99 240160 | B     | 4019 | 4019 | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 2001        | M01 240630 | B     | 1867 | 1867 | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 2001        | M01 241604 | B     | 3009 | 3009 | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 2001        | M01 242298 | B     | 286  | 286  | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 2001        | M01 242525 | B     | 1867 | 1867 | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 2007        | M07 240677 | B     | 1867 | 1867 | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 2001        | M01 242086 | B     | 1867 | 1867 | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 2001        | M02 240269 | B     | 1867 | 1867 | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 2006        | M06 240136 | B     | 286  | 286  | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 1975        | M98 252404 | B     | 456  | 456  | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 1998        | M98 253393 | B     | 1276 | 1276 | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 1998        | M98 253573 | B     | 1276 | 1276 | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |
| 1999        | M99 240371 | B     | 1276 | 1276 | ND   | ND   | ND   | ND   | ND   | ND   | CSF     | Meningitis/sepsis, 44 yrs |

a NA indicates that the cc belongs to the proposed cc286.
b f/l, full length.
c Site from which the isolate was recovered. N/p, nasopharynx; T/asp, tracheal aspirate; K/asp, knee aspirate; CSF, cerebrospinal fluid; NK, not known.
d ND, not detected.
e ?UTI, suspected urinary tract infection.

Of note, fHbps belonging to the same variant group share over 85% amino acid similarity, while there is only 60 to 70% similarity between the three variant groups (1, 33). fHbp is also an antigen that elicits serum bactericidal antibody responses in immunized individuals and is a key component of investigational vaccines for the prevention of meningococcal disease, in particular that caused by serogroup B, that are currently being evaluated in clinical trials (12). Immunization of mice with fHbps from variants 2 and 3 generates responses with some degree of immunological cross-reactivity, but these variants do not induce bactericidal antibodies against strains expressing V1 fHbp (32). The vaccine being tested by Pfizer consists of two fHbp protein subvariants (one V1 and one V3) (13), while the Novartis vaccine contains outer membrane vesicle (OMV) and recombinant antigens, including a chimeric protein consisting of a V1 fHbp fused to another protein (14).

During a recent genotypic analysis of potential vaccine antigens, we identified strains from individuals with invasive meningococcal disease in which fHbp either contained a frameshift mutation or was entirely absent; these isolates were likely to be deficient in expression of this important virulence factor and vaccine antigen. Here we present a characterization of the abilities of these strains to express fHbp and their capacities to bind fH.
swab and adjusted to an absorbance of 0.1 at 650 nm. A 1-ml aliquot of the suspension was then incubated at 60°C for 70 min to ensure killing of the bacteria. The cells were then pelleted at 6,000 g for 10 min, and DNA was extracted with the DNeasy blood and tissue kit (Qiagen, Crawley, United Kingdom) according to the manufacturer’s protocol. DNA elution was from columns and stored at 4°C.

PCRs were performed using the HotStarTag DNA polymerase kit (Qiagen) or Expand Hi-Fidelity polymerase (Roche). Routine PCR and sequencing protocols for fHbp were performed as previously described (27). Where necessary, primers targeting sequences within flanking genes were used (1869-2F, GAAG AAATCGTCGAAAGGCATCAAAC; 1871-Ralt, ATGCCGATACGCAGTTCCGC
cG/CJGTAACA), and PCR mixtures contained 2.5 μl of 10× PCR buffer, 2.5 μl of each primer (5 μM stock), 0.5 μl deoxynucleotide triphosphate (dNTP) mix (10 mM for each dNTP), 0.125 μl HotStarTag (Qiagen), 14.675 μl molecular-grade water, and 2 μl of eluted DNA template. Thermocycling conditions comprised an initial step of 96°C for 15 min, followed by 35 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 45 s. Amplified products were purified using ExoSAP-IT (USB Corporation) or the Qiagen PCR cleanup kit followed by cloning into pGEMTeasy (Promega). Sequencing reactions were performed using the BigDye v3.1 kit (Applied Biosystems). Sequencing reactions comprised 1.75 μl 5X sequencing buffer, 0.5 μl BigDye master mix, 0.66 μl primer (5 mM stock), 6.09 μl molecular-grade water, and 1 μl purified PCR product. Sequence analyses were performed on a 3130xl sequence analyzer (Applied Biosystems). Contig assembly and manual adjustment of bases were performed using Sequencher v4.8 (Gene Codes Corporation).

ELISAs for detection of fHbp. Bacteria were cultured overnight in medium, resuspended in phosphate-buffered saline (PBS), and then fixed in the presence of 3% paraformaldehyde for 1 h. Cells were resuspended to a final absorbance of 0.2 at 650 nm in bicarbonate buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate; pH 9.6), and 50 μl of 2 M NaOH to each well, and the optical density (OD) was read at 405 nm on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA).

RESULTS

The fHbp polymorphisms ΔT366 and ΔA650 are associated with loss of fHbp expression. Without the signal sequence, the open reading frame (ORF) of V1 fHbp typically ranges from 765 to 789 bp in length and encodes a protein consisting of 255 to 263 amino acids. Initially we identified a strain of N. meningitidis strain 8013 genome, FM99788; N. meningitidis strain ATCC 49260 genome, CP001104; N. meningitidis strain Z2491 genome, NC_003116; N. meningitidis strain FAM18 genome, NC_008767; N. meningitidis strain ATCC 49260 genome, NC_001120; N. meningitidis strain a110 genome, CP001156; N. meningitidis strain MC85 genome, NC_003112; Neisseria gonorrhoeae strain NCP10 genome, CP001050; N. gonorrhoeae FA 1090 genome, NC_009461; Neisseria lactamica strain ATCC 29370 whole-genome shotgun sequence ACEQ000008; N. lactamica strain Y92-109 whole-genome shotgun sequence, CACL01000005.

For detection of fH binding, following blocking in PBS/TMB, membranes were washed in PBS and then incubated with purified fH (5 μg/ml; Sigma Aldrich) in PBS/TMB for 2 h. After washing four times in PBS, membranes were incubated with goat polyclonal anti-human fH antibody (Calbiochem, EMD Biosciences), diluted 1:2,000 in PBS/TMB for 1 h. After further washes in PBS, membranes were incubated in murine anti-goat HRP-conjugated IgG (Sigma-Al
drich) diluted 1:10,000 in PBS/TMB for 45 min. Membranes were exposed to Hyperfilm (Amersham Biosciences).

Nucleotide sequence accession numbers. The following GenBank accession numbers were assigned to the sequences determined in this study: N. meningitidis strain 8013 genome, FM99788; N. meningitidis strain ATCC 49260 genome, CP001104; N. meningitidis strain Z2491 genome, NC_003116; N. meningitidis strain FAM18 genome, NC_008767; N. meningitidis strain ATCC 49260 genome, NC_001120; N. meningitidis strain a110 genome, CP001156; N. meningitidis strain MC85 genome, NC_003112; Neisseria gonorrhoeae strain NCP10 genome, CP001050; N. gonorrhoeae FA 1090 genome, NC_009461; Neisseria lactamica strain ATCC 29370 whole-genome shotgun sequence ACZQ000008; N. lactamica strain Y92-109 whole-genome shotgun sequence, CACL01000005.
ST-11 (cc11), except for one serogroup C strain which was an ST-7664 (cc11) isolate.

Sequence alignment showed that eight of the strains share the same \textit{fHbp} sequence, which is identical to allele 82 (peptide 78) in the databases of www.neisseira.org when the frameshift mutation is corrected. The \textit{fHbp} alleles from M05 240072 and 0030/01 differ from each other by just a single base (altering one amino acid, A22T) and from allele 82 by 5 or 6 nucleotides, with alterations of 2 (G30S and M35T) or 3 (A22T, G30S, and M35T) amino acids, respectively (Fig. 1A and B). Neither the 0030/01 nor the M05 240072 \textit{fHbp} subvariants had corresponding, nontruncated counterparts in the \textit{fHbp} or other sequence databases or among other isolates we have tested to date. However, the ST-11 isolate M01 240074 (\textit{fHbp} allelic subvariant 82) expresses an \textit{fHbp} that is identical to eight of the strains when the frameshift mutation is taken into consid-

FIG. 1. Alignments of \textit{fHbp}\textsubscript{\textit{DeltaT366}} alleles (A) and the predicted protein products (B), with the related, intact, allelic subvariant 82 (peptide 78). DeltaT366 (a) is the nucleotide and predicted amino acid sequence of isolates M07 241093, M08 240254, M08 240409, M08 240270, M98 253564, M00 242764, M00 242809, and M06 242760; delta T366 (b) represents isolate M05 240072; and delta T366 (c) shows sequences from isolate 0030/01. All isolates belong to cc11. Dots represent identical nucleotides/amino acids, and dashes represent gaps. The \textit{DeltaT366} residue is highlighted by a red ellipse. Nucleotide and amino acid differences are highlighted by green arrows.
peration and was therefore selected as a positive control for all isolates with the δT366 polymorphism in subsequent assays, while MC58/fHbp was used as a negative control.

To determine whether these strains express any detectable fHbp, we performed Western blot and whole-cell ELISA analyses with polyclonal sera raised against recombinant V1 fHbp (protein subvariant 1) (Fig. 2). Both methods demonstrated that, as with the negative control (MC58/fHbp), none of the isolates harboring fHbp with the δT366 polymorphism expressed detectable fHbp, with no truncated version of the protein detected by Western blot analysis. In contrast, fHbp was clearly expressed by the control strain M01 240074, which contains the intact subvariant 82 allele, as demonstrated by both ELISA and Western blot analyses. Therefore, the δT366 polymorphism is associated with loss of fHbp expression in these isolates.

An additional United Kingdom clinical isolate (M08 240219) was found to possess an fHbp gene with a different frameshift mutation, ΔA650, which is predicted to truncate the protein at amino acid 238, preceded by a series of 20 amino acids with no homology to fHbp (Fig. 3A). M08 240219 is a United Kingdom serogroup B clinical isolate belonging to ST-162 (cc162). This single cc162 isolate was identified among a total of 10 cc162 isolates from all invasive disease isolates in the epidemiological year 2007 to 2008 (frequency of the ΔA650 polymorphism in cc162 strains, 10%); no other strains from this clonal complex were examined. Aside from the frameshift, the fHbp allele in this strain is classified as V2, allele/peptide 21, which is expected to be expressed as a full-length protein by strains M08 240039, M08 240804, and M08 240374, all of which are clinical serogroup B, ST-162 isolates. Of note, Western blot analysis showed that fHbp was detected in whole-cell lysates of M08 240219 by using polyclonal sera raised against recombinant V2 fHbp (Fig. 3B), although the protein was of a lower molecular mass than that present in the control strain, M08 240039; this is consistent with M08 0240219 expressing a truncated version of fHbp. In contrast, no protein was detected on the surface of this strain by whole-cell ELISA (Fig. 3C), unlike results with the control strains M08 240039 and M08 240374.

**Identification of strains lacking an fHbp locus.** As well as strains with frameshift mutations, we identified seven serogroup B clinical isolates from which the fHbp gene could not be amplified by PCR using primers targeting sequences flanking the fHbp ORF or highly conserved regions within the gene.
itself (data not shown). Initially, a single \(fHbp\)-negative isolate (M07 240677; ST-1867) was identified from the epidemiological year 2007 to 2008, from which all invasive disease isolates (\(n = H11005\) 613) underwent MLST and \(fHbp\) characterization. To determine whether this was an isolated occurrence, further isolates belonging to ST-1867 and related STs (i.e., cc286 (Fig. 4A) were sought for \(fHbp\) characterization. Among these, a single isolate (M06 240136) was identified from routine MLST between January 2005 and July 2007 inclusive (during which time all January and July isolates were subjected to MLST analysis). Two isolates (M01 240136) were identified from routine MLST data obtained from 1998 and 2002 inclusive (during which time approximately 1 in 10 isolates underwent MLST analysis). Two isolates (M01 240136, M99 240677, M99 241177, M01 240630, M01 241604, M01 242298, M98 253393, M98 253573, and M09 240371) were identified from study-related MLST data (M. Maiden, unpublished data) obtained from November 1998 to November 1999 and November 2000 to November 2001, when all invasive disease isolates (\(n = H11005\) 1,688 and \(n = H11005\) 1,289, respectively) underwent MLST. A single isolate (M98 252404, collected in 1975) was identified from 1975 and 1985, from which all isolates (total of 125 isolates in 1975) and a representative proportion of isolates (100 isolates in 1985) underwent MLST, respectively. No further isolates with these, or related, STs were identified among the data sets outlined. Among these 14 isolates identified as belonging or related to ST-1867, 7 (50%) did not harbor an \(fHbp\) allele, while the remainder contained intact \(fhbp\) alleles (5 V3 and 1 each of V1 and V2). Alignments of sequences from these seven strains with those from the seven isolates from which we were unable to amplify an \(fHbp\) PCR product failed to reveal homology across a central region spanning 1,161 bp (Fig. 4B); this region is flanked by regions of high sequence identity (88.5% to 94.2%). Similar results were obtained in a BLASTn search of the nucleotide database that returned partial alignments among seven meningococcal genomes (strain 8013 [37], a14 [41], Z2491 [35], FAM18 [3], 053442 [36], \(fH9251\) 710 [22], and MC58 [46]) and two gonococcal genomes (strains NCCP11945 [8] and FA 1090 [L. A. Lewis et al., unpublished data]). The recently published 020-06 \(N.\ lac\) tamica genome (2), however, gave alignments with 94% coverage and >94% identity. Similarly, a BLASTn search of the
FIG. 4. (A) The meningococcal ST-286 cluster as deduced by eBURST analysis of the Neisseria spp. MLST database (pubmlst.org). Numbers show STs in the cluster (defined as sharing four identical alleles out of seven from MLST), with those in bold type, including isolates lacking the fHbp locus. The blue dot represents the predicted founder ST, ST-286. (B) Nucleotide alignment of fHbp-negative cc286-related isolates (M07 240677 and M01 241604), fHbp-deficient N. lactamica isolates (020-06, ATCC 23970, and Y92-1009), fHbp+/H11001 cc286-related isolates (M06 240136, M01 242006, M98 240371, M99 240160, and M99 241177) have identical sequence with M07 240677 in the regions illustrated. The remaining fHbp-deficient cc286-related isolates (M01 240630, M01 242298, M01 242525, M99 240160, and M99 241177) have identical sequence with M07 240677 in the regions illustrated. The shaded region represents 1,152 bp in which there is no homology between the fHbp” (highlighted in red) and fHbp-deficient (highlighted in green) isolates.

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whole-genome shotgun reads database returned two partial N. lactamica genomes (strains ATCC 23970 [L. Fulton et al., unpublished data] and Y92-1009 [T. E. Vaughan et al., unpublished data]) with 93 to 99% coverage and >95% identity (Fig. 5). This indicates a probable horizontal transfer event between the two species. Interestingly the 1,200-bp region contained an ORF of between 739 and 899 bp in the opposite orientation to \( fHbp \). The ORF, annotated as a putative opacity protein in the N. lactamica strain 02-06 genome, contains a tract of 5 to 25 GCGTTCCT repeats. Addition or subtraction of repeat units in the ORF alters the reading frame; two of the seven \( cc286 \)-related isolates harbor an in-frame ORF. A tBLASTn search of the translated ORF against the nucleotide collection database revealed up to 36% amino acid identity (99% query coverage) against the opacity protein (Opa) of various neisserial species.

ELISA and Western blotting results demonstrated that the strains harboring a deleted \( fHbp \) locus did not express \( fHbp \) when polyclonal antibodies raised against recombinant V1, V2, or V3 \( fHbp \) were used (Fig. 6); strains matched for ST and expressing \( fHbp \) were used as controls (i.e., M01 242006 [ST-1867], M02 242629 [ST-1867], and M06 240136 [ST-286]) wherever possible.

**Analysis of \( fH \) binding to strains not expressing \( fHbp \).** \( fH \) bound to the surface of the meningococcus retains its activity as a cofactor for \( fH \)-mediated cleavage of C3b and enhances survival of *N. meningitidis* in the presence of the human complement system (38). Therefore, we examined whether the strains with frameshift mutations in \( fHbp \) or lacking the entire \( fHbp \) gene bound \( fH \). Far Western analysis was used to detect binding of full-length \( fH \), as this method can differentiate between interactions with truncated \( fHbp \) and other potential targets, such as the 17-kDa protein NspA (24). There was no evidence of \( fH \) binding to the strains with the \( \Delta T366 \) mutation. Far Western analysis was used to detect binding of full-length \( fH \), as this method can differentiate between interactions with truncated \( fHbp \) and other potential targets, such as the 17-kDa protein NspA (24). There was no evidence of \( fH \) binding to the strains with the \( \Delta T366 \) mutation, even though binding was detected to the control strains expressing full-length \( fHbp \) (Fig. 7A). Similarly, no \( fH \) binding was detected to M08 240039, which has the \( \Delta A650 \) polymorphism (Fig. 7B); of note, even though the control strains M08 240039 and M08 240374 expressed \( fHbp \) based on Western analysis and ELISA, this protein did not bind \( fH \) in the far Western analysis (Fig. 7B). However, in lysates of M08 240374, far Western blotting detected a faint band of around 17 kDa, consistent with NspA; \( nspA \) was detected by PCR in all strains not expressing \( fHbp \) (data not shown). There was no \( fH \) binding detected in strains with the \( fHbp \) locus deleted (Fig. 7C).

**DISCUSSION**

The ability of certain bacteria to avoid elimination by the immune system is critical to their success as pathogens. Here we report the characterization of serogroup B and C meningococcal strains with polymorphisms predicted to result in truncations or a complete lack of \( fHbp \). These isolates retain their capacity to cause disease despite their failure to express a functional \( fHbp \) for recruiting \( fH \), the key negative regulator of the alternative complement pathway.

*N. meningitidis* is a highly diverse bacterium that expresses 1 of 12 capsular serogroups, each with a distinct chemical composition. Examination of the complete genome sequences of the meningococcus reveals that it possesses multiple mecha-

isms that enable phenotypic variation. These include homoplastic tracts that mediate phase variation of surface antigens, including lipopolysaccharide, repeat sequences that can undergo recombination (such as DNA uptake sequences and Corrêa elements), and transposable elements (42). This variation enables the bacterium to circumvent killing by the immune system, and it is likely that different lineages of the pathogen have evolved distinct mechanisms to survive in the hostile environment of the host.

While a recent epidemiological analysis detected strains with a single point mutation that leads to a potential truncation of a V1 \( fHbp \) (18), we have described isolates from three distinct clonal complexes of the meningococcus, cc11, cc162, and those centered on ST-286, in which \( fHbp \) contains a frameshift mutation or has been replaced (along with flanking regions) by sequences shared with the commensal species *N. lactamica*. As all these isolates were obtained from patients with disseminated meningococcal disease, this demonstrates that \( fHbp \) is not essential for pathogenesis of these strains, even if recruitment of \( fH \) contributes to meningococcal survival in serum and human blood (38, 43). We could not detect \( fH \) binding to any of these strains except M08 240374 by far Western analysis using purified \( fH \) and available polyclonal antibodies. This method has been used to successfully detect \( fH \) binding to meningococci either via \( fHbp \) or other potential ligands (24, 38) and makes it possible to exclude nonspecific associations (by performing blotting assays in the absence of \( fH \) or the primary antibody).

In the absence of \( fHbp \), *N. meningitidis* could recruit \( fH \) by alternative receptors. It was shown recently that certain meningococcal strains can bind \( fH \) to their surface via NspA, which has been evaluated as a vaccine antigen (30). NspA binds \( fH \), especially in the absence of capsule and LOS sialylation, and can promote bacterial survival in dilute human serum (i.e., 1.5 to 5%) in strains expressing low levels of \( fHbp \) (24). We did detect \( fH \) binding to a protein of the same molecular mass as NspA in M08 240374 by far Western analysis. It is possible that the other strains utilize distinct complement regulators to promote their survival during bloodstream infection. For instance, it has been reported that C4BP, the negative regulator of the classical pathway, is also bound by *N. meningitidis*; we did not examine the strains for binding to C4BP, as this regulator is not recruited by the meningococcus in any appreciable amounts at physiologically relevant osmolarities (21). However, it is possible that the strains compensate for the lack of \( fHbp \) by recruiting other complement regulators.

cc11 is a hyperinvasive lineage associated with relatively high levels of mortality and morbidity, has caused outbreaks of serogroup C and W135 disease affecting individuals in developed and developing countries, and is not commonly recovered from healthy individuals in carriage studies (29). Of note, cc11 strains now account for approximately 18% of serogroup B disease in North America (19). A recent survey showed that cc11 strains from across the world express a diverse range of \( fHbp \)s belonging to any of the three variant groups (1). However, the subset of cc11 strains identified here have the same frameshift mutation and are predicted to express V1 \( fHbp \)s that are either identical to each other or differ by 3 amino acids at the most when the frameshift is taken into account. Therefore, it is likely that these alleles originated following a single
genetic event in this hyperinvasive lineage, even though the isolates were obtained from geographically distinct locations (United Kingdom and the Czech Republic) over an 11-year period. In England and Wales in 2007 to 2008, 4 out of 22 cc11 strains were found to have fHbp alleles with \( /H9004_T366 \), representing a significant proportion (18.2%) of strains of this important hypervirulent lineage.

We recently characterized three serogroup C ST-11 strains from Spain that produce high levels of capsule due to the presence of an insertion sequence, IS\(1301 \), in the capsule biosynthesis locus (\(cps\)), which promotes resistance against complement-mediated lysis (47). A diverse collection of other meningococcal isolates demonstrated that this genetic change is also found in a significant proportion of cc269 and other isolates. However, in cc269 this polymorphism is almost invariably accompanied by other changes in the \(cps\) that counter the effect of IS\(1301 \) on capsule biosynthesis (23). Therefore, the cc11 strains may have particular mech-

FIG. 5. Nucleotide alignments of the region shared by \(fHbp\)-deficient cc286-related isolates and the \(fHbp\)-deficient \(N. lactamica\) isolates (020-06, ATCC 23970, and Y92-1009). The arrow highlights the open reading frame of a putative phase-variable opacity protein. Dots represent identical nucleotides, and dashes represent gaps. The putative ORF is bounded by red lines, with an arrow to denote the start codon. The longest GCCGTTTCTT repeat tract (isolate M07 240677) is bounded by blue lines with an arrow showing the repeat unit. The final repeat unit in the other isolates is denoted by a blue forward slash.
anisms for subverting complement-mediated killing, allowing fHbp to be dispensable.

c162 strains account for a small but consistent proportion (5 to 10%) of serogroup B cases in developed countries, such as those in Europe and the United States (19), and are not frequently found in carriage studies (M. Maiden, personal communication). Although little is known about the features of c162, it is interesting that several strains lack a functional two-partner secretion (TPS) system, through a frameshift mutation in the gene encoding the cognate transporter TpsB (49), which is responsible for transporting TpsA across the other membrane. The TPS system contributes to the adhesion of the bacterium to epithelial cells through unknown mechanisms.

We identified a single strain from this clonal complex, M08240219, with a frameshift mutation that truncates the protein prior to the site corresponding to a critical amino acid in V1 proteins, Glu304, which is a Thr in V2 and V3 fHbps (40). It is also apparent that the truncated form of the protein does not reach the cell surface, thus abrogating further a potential role in recruiting fH. As we found only one strain with this change, it is possible that the mutation represents an isolated occurrence and/or occurred during its isolation or passage while being sent to the Meningococcal Reference Unit. However, of note, the fHbp expressed by the control strains (i.e., peptide 21) does not bind fH by far Western analysis, although the basis of this is being investigated.

Initially a single isolate with an absent fHbp locus (M07240677; ST-1867) was identified from all disease isolates (n = 613) in the epidemiological year 2007 to 2008, which all underwent MLST and fHbp characterization. To determine whether this was unique, isolates belonging to ST-1867 and related STs (centered around ST-286 [Fig. 4A]) were sought to determine whether they contained fHbp. Among the 14 ST-286-related isolates identified from our collection spanning 35 years, 7 had the deleted allele, representing STs 286, 1867, 3309, and 4019; disease caused by ST-286-related strains is infrequent. Nonetheless, we found that 50% of isolates lacked fHbp, with the locus replaced by sequences which are also found in N. lactamica strains and are likely to have been acquired by horizontal transfer between this species and N. meningitidis. Interestingly, this sequence includes an open reading frame that is predicted to encode a protein related to the opacity proteins. Of the opacity proteins, the meningococcal Opas are phase-variable proteins that have been shown to be

![Fig. 7](image-url)
the target of the activated complement factors C4b and C3b (25), although there is no evidence that they recruit regulatory proteins. It is not clear what is the function of the additional opacity protein in the ST-1867 strains, although it appears to be a phase-variable antigen, due to the presence of a repeat sequence in its ORF. Of note, the opacity protein Opc binds vitronectin (50), which is a negative regulator of the complement system (4). Therefore, it is possible that expression of Opa can compensate for the lack of fHbp in these strains.

fHbp is an important antigen for two investigation vaccines against serogroup B N. meningitidis currently undergoing advanced clinical trials. The occurrence of disease strains lacking fHbp is reminiscent of strains lacking other vaccine candidates, such as PorA and FetA (9, 34), and has implications for vaccines containing these antigens. However, cc162 and cc286 strains are not frequent causes of meningococcal disease in the United Kingdom, while cc11 has been mostly associated with serogroup C disease, which is preventable by the MenC conjugate vaccine. Altogether, the fHbp-nonexpressing strains account for 0.6% of MenB disease isolates and 1% of all isolates in the United Kingdom based on the last year for which we have complete MLST data. Just under 7% of MenB isolates in the United States between 2000 and 2005 belong to corresponding cc’s and so may also include fHbp-deficient lineages (19). Therefore, strains lacking fHbp would have relatively little impact on the efficacy of the vaccines being evaluated. However, the occurrence of fHbp-deficient strains capable of causing invasive meningococcal disease does illustrate that active surveillance of disease and carriage strains is vital throughout and following vaccine implementation, in order to detect the potential emergence of strains that have evolved under selective pressure of any vaccine to no longer express this antigen.

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