The effect of iron availability on transcription of the *Neisseria meningitidis* *fHbp* gene varies among clonal complexes

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Factor H binding protein (*fHbp*) is a major antigenic component of novel vaccines designed to protect against meningococcal disease. Prediction of the potential coverage of these vaccines is difficult, as *fHbp* is antigenically variable and levels of expression differ among isolates. Transcriptional regulation of the *fHbp* gene is poorly understood, although evidence suggests that oxygen availability is involved. In this study iron accessibility was found to affect *fHbp* transcription. However, regulation differed among meningococcal clonal complexes (ccs). For the majority of isolates, increased iron concentrations upregulated transcription. This effect was enhanced by the presence of a 181 bp insertion element upstream of *fHbp*, associated with isolates belonging to cc4 and cc5. Conversely, meningococci belonging to cc32 showed iron-repressed control of *fHbp*, as regulation was dominated by cotranscription with the iron-repressed upstream gene *cbbA*. These results highlight the complexity of *fHbp* regulation and demonstrate that control of transcription can vary among genetic lineages.

INTRODUCTION

*Neisseria meningitidis*, the meningococcus, is a major cause of bacterial meningitis and septicaemia. Invasive meningococci typically express one of five capsular polysaccharides, A, B, C, W135 and Y, which are used to type them into serogroups (Jolley et al., 2007). Effective protein–polysaccharide conjugate vaccines are available against serogroup A, C, W135 and Y meningococci (Snape & Pollard, 2005); however, because of its similarity to glycosylated human antigens, the serogroup B polysaccharide is widely regarded as a poor vaccine candidate (Yongye et al., 2008). Instead, the development of a vaccine with the potential to protect against organisms expressing the group B capsule has focussed on subcapsular protein antigens. One such antigen is factor H binding protein (*fHbp*), a surface-exposed lipoprotein that is a major component of two vaccine candidates: Bexsero (Novartis) and rLP2086 (Pfizer). Both are at an advanced stage of clinical development and have been shown to induce *fHbp*-specific bactericidal antibodies in humans (Findlow et al., 2010; Halperin et al., 2010).

As its name indicates, *fHbp* binds human factor H (*fH*), a key negative regulator of the alternative complement pathway. Binding *fH* enables the meningococcus to evade killing by human complement, and thereby enhances its survival *in vivo* (Madico et al., 2006). However, the expression of *fHbp* varies several fold among invasive isolates, affecting its potential as a target for bactericidal antibodies. This has implications both for *fHbp* as a target antigen for vaccination and for the evaluation of anti-*fHbp* serological responses *in vitro* (Giuliani et al., 2010) using tests such as the serum bactericidal assay (SBA) or the meningococcal antigen typing system (MATS) (Donnelly et al., 2010). Based on microarray data it has been suggested that the *fHbp* gene is a member of the FNR (fumarate and nitrate reductase) regulon and that its expression is therefore subject to oxygen availability (Bartolini et al., 2006). Under anaerobic conditions, FNR is present as a dimer containing an iron–sulphur cluster. The FNR dimer binds to consensus sequences in the promoters of various genes and enhances transcription. In the presence of oxygen, the iron–sulphur cluster is degraded and the dimer loses its ability to bind DNA, resulting in reduced transcription of upregulated genes.
(Edwards et al., 2010). Recent evidence shows that fHbp is expressed from two independent transcripts, one of which is under the control of a promoter that responds to oxygen limitation in an FNR-dependent manner (Oriente et al., 2010). In addition, upstream of the fHbp promoter sequence in the Z2491 genome there is a nucleotide sequence that has similarities to a fragment of an insertion element (IE). This nucleotide sequence is not present upstream of the fHbp gene in other published genomes, but whether it affects the expression of fHbp is currently unknown (Oriente et al., 2010). Although the role of fHbp in binding complement factor H is well established, other possible functions of this protein have been identified. For example, fHbp has been shown to be important for survival of meningococcal cells in the presence of the antimicrobial peptide LL-37 (Seib et al., 2009) and to bind ferric enterobactarin in vitro (Rappuoli, 2010). Initial characterization of fHbp also showed sequence and structural similarity to the meningococcal transferrin binding proteins (Cantini et al., 2006; Massignani et al., 2003).

In this study, the transcription levels of fHbp were compared across 90 meningococcal isolates grown in iron-restricted and iron-sufficient media. Isolates were from the Multi-Locus Sequence Typing (MLST) Neisseria reference set (Maiden et al., 1998), including both disease and carriage isolates collected between 1937 and 1996 from a variety of clonal complexes (ccs) and geographical locations. Furthermore, the availability of the whole-genome sequences allowed the fHbp gene and upstream sequence to be compared among the isolates (H. B. Bratcher and others, unpublished results). The study revealed differences in the transcription of fHbp among different ccs of the meningococcus and raised further questions about the evolved functions of this protein.

**METHODS**

**Isolates.** N. meningitidis isolates investigated were from the published MLST reference set (Maiden et al., 1998). Information on all isolates is listed in Supplementary Table S1.

For all 107 isolates, sequences of fHbp, cbbA and the intergenic (IG) region between these genes were from the BIGSdb (Jolley & Maiden, 2010), hosted in the Department of Zoology at the University of Oxford, using the BLAST function. Sequences were aligned and compared using DNASTAR Lasergene (v. 8.0.2) software.

MLST allelic profile data were also downloaded from the BIGS database (Jolley & Maiden, 2010). A majority-rule consensus tree depicting the phylogeny of the isolates was obtained from Xavier Didelot (University of Oxford) (Didelot et al., 2009) and edited using MEGA version 5 software.

**Isolation of total RNA.** Nine isolates from the MLST reference collection were not available for RNA extraction. RNA was prepared from the remaining 98 isolates. Bacteria were resuspended from overnight growth on blood agar plates (Oxoid) into Mueller–Hinton broth (MH; Oxoid) or MH depleted of available iron by the addition of 50 µM deferoxamine mesylate (DFAM) salts (Sigma Aldrich) as a chelator. After resuspension to OD<sub>600</sub> 0.18–0.22, meningococci were grown at 37°C with 150 r.p.m. rotational shaking for 5 h. To protect the RNA from enzymic degradation the culture was mixed with RNAprotect Bacteria reagent (Qiagen), in a ratio of one volume culture to two volumes reagent, and incubated at room temperature for 5 min. Bacterial cells were harvested by centrifugation at 9600 g for 10 min and the resulting cell pellets were frozen overnight at −80°C. Total RNA was extracted from the cell pellets using an RNaseasy Mini kit, and DNA was removed by on-column RNase-free DNase digestion (Qiagen). Total RNA was eluted in 50 µl RNase-free water.

**Quantitative real-time PCR (qRT-PCR) assay.** Custom primers (Thermo Fisher) and probes (TIBMolbiol) used for gene expression assays are listed below. Fluorescent markers used were 6OE (6-carboxy-4,5′-dichloro-2′,7′-dimethoxyfluorescein) and 6FAM (fluorescein). BlackBerry Quenchers (BBQ) were used on all probes.

Primers used for gdh were gdh350F (5′-TGCCATTAAAGCGCA-AATC-3′), gdh417R (5′-CTTGCGGATCGCGGTAGA-3′) and gdh374T (5′-6OE-A CGAACGCTGGAGGCGTTC-BBQ-3′). Primers used for fHbp (assay 1) were fHbp117F (5′-CTGCGAGAAAACGGGACGAC-3′), fHbp1185R (5′-CTGGTCGGCTTTTTCCAT-AAG-3′) and fHbp142P (5′-6FAM-CTGGCGGCAAGGTTGCGG-BBQ-3′). Primers used for fHbp peptide 1 (assay 2) were fHbp407F (5′-GGAGCACAAATGCACCTGTA-3′), fHbp468R (5′-CCGC-GTCCCCGGCATA-3′) and fHbp435P (5′-6FAM-CAAGAAGCGGCA-GGGCGAC-BBQ-3′). Primers used for cbbA were mmb1869F (5′-GGGCGAC-BBQ-3′), mmb1869R (5′-GGGCGAC-BBQ-3′) and fHbp142P (5′-CCGC-GTCCCCGGCATA-3′) and mmb1869P (5′-6FAM-CATCGCCCAAC-TGTTGATCATGC-BBQ-3′). Amplicon sizes were as follows: gdh, 69 bp; fHbp assay 1, 70 bp; fHbp assay 2, 62 bp; cbbA 72 bp.

Primer and probe sequences were determined using Primer Express 2.0 software (Applied Biosystems). mRNA from the majority of isolates (82 isolates in total; Supplementary Table S1) was detected by a single assay (assay 1). However, due to sequence variation, fHbp peptide 1 (variant 1.1 according to the MSTR typing scheme) was amplified with a fHbp variant-specific primer/probe set (assay 2, eight isolates). The fHbps from these isolates were not amplified by either set of primers and probes, and were thus omitted from further investigation (Supplementary Table S1).

RT-PCR assays were completed in MicroAmp Fast Optical 96-Well Reaction Plates sealed with MicroAmp Optical Adhesive Film (both Applied Biosystems). Expression of the housekeeping gene gdh was used as an internal control in each well. For both fHbp and cbbA, reactions were mixed to a final volume of 23 µl with reagents at the following final concentrations: 1× TaqMan RT Enzyme Mix, 1× TaqMan RT-PCR Mix (both from the TaqMan RNA-to-CT 1-Step kit, Applied Biosystems), 300 nM each target and control primers, 200 nM target probe and 200 nM gdh probe. A 2 µl volume of total meningococcal RNA was added to each reaction. A negative RNA-free water. Positive control sample was set as the 'calibrator sample' on each plate. All reactions were run in triplicate.

RT-PCR was completed using an Applied Biosystems 7500 Fast RT-PCR system with the following thermocycling conditions: 48°C for 15 min; 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 60 s. Fluorescence was recorded at the end of each extension step. Relative quantity (RQ) values were calculated with the 2<sup>−ΔΔCt</sup> method by the Applied Biosystems 7500 Fast System Sequence Detection software. The gdh reaction was used as an endogenous control. The H44/76 positive control sample was set as the 'calibrator sample' on each plate, to which all RQ values were normalized.

Detection of bicistronic transcript. To detect transcription of the upstream IG region, the primers IGF (5′-A CAAAATGCGCT- GAAC-3′) and IGR (5′-CATCAATGAGGCGAGTGCA-3′) were used.
in an RT-PCR assay with total RNA as described above using the SYBR Green RNA-to-CT 1-Step kit (Applied Biosystems). The *gdh* primers were used in a separate reaction for each RNA sample as a positive control. Thermocycling conditions were as described above, and the fluorescence released by the SYBR Green dye was recorded at the end of each extension step. Following amplification, PCR products were resolved at 100 V for 2 h on acrylamide TRIS-borate-EDTA (TBE) gels containing: 13 % (v/v) bis-acrylamide (29 : 1, Bio-Rad), 1 x TBE, 0.16 % (w/v) ammonium persulphate (NBS Biologicals) and 0.08 % (v/v) N,N,N',N'-tetramethylene-diamine (TEMED; Bio-Rad). Gels were incubated in 1 x TBE containing 0.005 % (v/v) Safeview Nucleic Acid Stain (NBS Biologicals) for 20 min prior to detecting the bands under UV light. Successful amplification was indicated by the presence of bands corresponding to amplicons of 69 bp for both the IG region and *gdh*.

**RESULTS**

**Comparison of *fhbp* upstream regions**

The nucleotide sequences of *fhbp* and its upstream region in all 107 MLST reference isolates were extracted from the BIGSdb (Jolley & Maiden, 2010) and aligned using DNAstar Lasergene MEGALIGN (v. 8.0.2) software. In 64 of the isolates analysed the upstream region was highly conserved, with >87.6 % sequence identity. However, in 43 of the investigated isolates there was a 181 bp IE upstream of the coding region. This IE was observed in a single meningococcal isolate by Oriente et al. (2010), and was predicted to be positioned downstream of the −10 region (Oriente et al., 2010) (Fig. 1). The IE was identified in all isolates belonging to sequence types (STs) 1, 4 and 5 (Fig. 2). Further scrutiny of the sequence data from the reference isolates in BIGSdb revealed that the IE was present at alternative positions among the different genomes rather than upstream of the *fhbp* gene, often occurring at multiple sites within the genome. The location of the IE varied among isolates, although many isolates contain the sequence between the convergent ORFs *mtrR* and *nmb1718*.

**Effect of iron availability on *fhbp* transcription levels**

To investigate the effect of iron on *fhbp* transcription, levels of *fhbp* mRNA extracted from isolates grown under iron-replete and -restricted conditions were determined using qRT-PCR. In the majority of isolates tested (assay 1, 82 isolates in total), levels of mRNA were significantly higher under iron-replete conditions than iron-restricted conditions (*P*<0.001, Fig. 3). This effect was more pronounced in isolates containing the IE upstream of the gene than in those without the IE in this position (*P* = 0.007, Fig. 3). In strains containing the IE the mean RQ natural log under iron-replete conditions was 0.578 ± 0.139 versus 0.034 ± 0.108 under iron-restricted conditions (*P*<0.001). In comparison, for strains not containing the IE, the mean RQ natural log under iron-replete conditions was 0.226 ± 0.160, versus −0.076 ± 0.72 under iron-restricted conditions (*P* = 0.034).

Conversely, most isolates belonging to cc32 produced significantly less *fhbp* mRNA under iron-replete conditions than iron-restricted conditions (mean RQ natural log = 0.299 ± 0.164 under iron-replete conditions versus 0.166 ± 0.142 under iron-restricted conditions, *P* = 0.001) (Fig. 4a). One cc32 isolate, NG 080, showed very low *fhbp* transcription levels, regardless of iron availability.

**Co-regulation of *fhbp* and *cbbA***

In the meningococcal isolate MC58, belonging to cc32, *fhbp* is expressed as part of a bicistronic transcript from the upstream gene *cbbA* (Oriente et al., 2010). To investigate this observation further, PCR was used to amplify the IG region between *cbbA* and *fhbp* from total RNA extracted from all eight cc32 isolates in the reference panel as well as from nine diverse isolates representing other ccs and *fhbp* alleles, including four isolates that

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**Fig. 1.** Genomic context of *cbbA* and *fhbp*, showing the position of the 181 bp IE relative to the *fhbp* coding region in the Z2491 genome sequence. The positions of the FNR-binding sequence (FNR box) and −35 and −10 regions (labelled), as determined elsewhere (Oriente et al., 2010), are shown. The RNA transcripts (both monocistronic and bicistronic) and directions of oxygen regulation demonstrated in meningococcal strain MC58 are also shown.
contained the IE upstream of fHbp. SYBR Green dye was used to follow the amplification of cDNA during the RT-PCR, and PCR products were subsequently electrophoresed on an acrylamide gel to verify that PCR products of the expected size were present. The positive control template gdh was successfully amplified from all the RNA samples tested. The amplification of the IG region suggested that the ability to produce a bicistronic transcript was restricted to isolates of cc32: no isolates tested from other ccs produced a PCR product of the expected size (69 bp, Fig. 5). A single cc32 isolate in this set, NG 080, did not show the presence of the intergenic transcript.

To investigate whether the gene situated upstream of fHbp, cbbA, is also iron-regulated, the level of transcription following growth under iron-replete and -depleted conditions was measured using qRT-PCR. The cc32 isolates present in the isolate collection exhibited significant iron restriction of cbbA transcription [mean RQ natural log 0.237 ± 0.127 under iron-replete conditions versus 0.499 ± 0.130 under iron-restricted conditions, P=0.006; Fig. 4(b)]. Isolates tested from other ccs showed iron-repressed transcription of cbbA similar to that of cc32 isolates (data not shown). For isolates in which expression of the intergenic transcript was detected, regression analysis showed a significant linear correlation between RQ values obtained for fHbp and cbbA [P=0.002; Fig. 4(c)].

Fig. 2. Distribution of the presence of the IE upstream of the fHbp gene across the ccs present in the isolate set investigated. The tree shows the majority-rules consensus relationships between all isolates when using 20 housekeeping gene fragments, as determined previously (Didelot et al., 2009). Each terminal node on the tree corresponds to a single isolate. Isolates are shown as either containing the IE (●) or not containing the IE (□). ccs containing more than five isolates in the panel are boxed.

Fig. 3. Comparison of the effect of iron availability on fHbp transcription levels in all 82 isolates tested using assay 1. Error bars, upper 95% confidence intervals of the mean. Due to the use of different assays, isolates expressing fHbp variant 1.1 (eight isolates, all cc32) could not be directly compared with other isolates and were analysed separately. When grown in iron-restricted media (Fe−, grey bars), fHbp RQ values were significantly lower than when grown in iron-sufficient media (Fe+, black bars). This effect was highly significant in isolates containing the upstream insert (P<0.001), but the difference was reduced in isolates without the insert (P=0.034).
Fig. 4. Comparison of the effect of iron availability on fHbp and cbbA transcription levels in all isolates belonging to cc32 (eight isolates in total, expressing fHbp variant 1.1). Cultures were grown in iron-restricted and iron-replete media on three separate occasions. Bars show the mean RQ values for all RNA extractions (Fe−, grey bars; Fe+, black bars); error bars, upper 95% confidence intervals of the mean. (a) Effect of iron availability on fHbp RQ values. A single isolate, NG 080, was excluded from the analysis of fHbp as the RQ values for this isolate were very low in comparison with other cc32 isolates (mean RQ natural log −3.552 under iron-replete conditions, −3.827 under iron-restricted conditions). When grown in iron-restricted media, fHbp RQ values were found to be higher than when grown in iron-sufficient media. When tested by two-sample t test, excluding NG 080, the effect of iron on fHbp RQ values was found to be significant (P = 0.001). (b) Effect of iron availability on cbbA RQ values in all cc32 isolates. When grown in iron-restricted media, cbbA RQ values were found to be significantly higher than when grown in iron-sufficient media (P = 0.006). (c) Correlation between transcription of fHbp and cbbA in cc32 isolates. RQ values from Fe+ (▲) and Fe− (○) cultures. There was a significant correlation between fHbp RQ and cbbA RQ values of cc32 isolates (P = 0.002), with the exception of NG 080.

Fig. 5. PCR products from amplification of cDNA of the IG region upstream of fHbp, with amplification of gdh (69 bp) as a positive control. Control reactions were also run in the absence of reverse transcriptase in order to detect contaminating DNA (IG RT−) and with genomic DNA to confirm that the appropriate sequence was present for primer binding (IG gDNA). The presence of a PCR product from amplification of the IG region (69 bp) suggests the expression of a bicistronic transcript. (a) Amplification from total RNA of the cc32 isolates in the reference isolates set; (b) amplification from total RNA of nine isolates from various ccs. Isolates F4698, 2059001, E26 and S5611 contained the IE.
DISCUSSION

The fHbp of \textit{N. meningitidis} is a component of two vaccines developed ostensibly to control serogroup B meningococcal disease (Donnelly \textit{et al.}, 2010; Jiang \textit{et al.}, 2010). The currently accepted correlate of protection used to evaluate such vaccine candidates is the ability to induce functional antibodies, as measured in an SBA (Borrow \textit{et al.}, 2006). To evoke an effective bactericidal response against the meningococcus, it is critical that fHbp is expressed on the surface of the bacterium (Koebelning \textit{et al.}, 2011), and there is evidence that the level of expression of the protein on the bacterial surface correlates with the susceptibility of target isolates to fHbp-specific antibodies in an SBA (Jiang \textit{et al.}, 2010). The level of fHbp expression \textit{in vitro} varies among meningococcal isolates. Furthermore, regulation of fHbp is likely to vary within the host, depending on levels of exogenous nutrients. Current evidence that oxygen availability plays a role in the regulation of fHbp expression, via the regulatory protein FNR, supports this assumption (Bartolini \textit{et al.}, 2006; Oriente \textit{et al.}, 2010). The findings of this and previous studies indicate that the regulation of fHbp is intricate and differs among ccs.

The availability of iron varies within the human host, and the meningococcus has evolved multiple mechanisms for the acquisition of iron from its host sources (Genco & Desai, 1996), which is reflected in the numerous meningococcal genes that have been shown to be iron-regulated (Grifantini \textit{et al.}, 2003). In this study, qRT-PCR measurements of the effect of iron availability on levels of transcription of the fHbp gene in a diverse collection of meningococci showed that, for the majority of the isolates, growth in iron-depleted conditions resulted in significantly lower levels of fHbp transcription than growth in iron-replete media. In contrast, cc32 isolates showed significantly higher levels of fHbp transcription when grown under iron-restricted conditions. This observation suggests that different regulatory mechanisms act upon this gene depending on the genotype of the isolate investigated.

Earlier work (Oriente \textit{et al.}, 2010) demonstrated that fHbp RNA in the cc32 isolate MC58 is present in two different transcripts: a transcript expressed from the fHbp promoter, and a bicistronic transcript also containing the upstream gene \textit{cbbA} (Oriente \textit{et al.}, 2010). Employing RT-PCR, the co-transcription of these genes was investigated across the collection of isolates, showing that the presence of the bicistronic transcript was confined to isolates belonging to cc32. Transcriptional termination in \textit{Neisseria} is thought to occur frequently at Rho-independent terminators (Kingsford \textit{et al.}, 2007), which are formed of palindromic loops in DNA secondary structure that prevent advancement of the polymerase, although the structure and stability of these loops is dependent on the DNA sequence present (Abe & Aiba, 1996). The sequences of the IG region between \textit{cbbA} and fHbp were compared among all isolates, and several nucleotide polymorphisms were detected in cc32. This sequence variation could result in a shorter or less stable palindromic loop, allowing DNA polymerase to read through the putative transcriptional terminator in cc32 isolates.

We have shown that the control of fHbp transcription in cc32 isolates grown in iron-restricted MH media was dominated by the \textit{cbbA} promoter. The \textit{cbbA} gene encodes a fructose-1,6-bisphosphate aldolase, a metabolic enzyme involved in glycolysis. In this bicistronic transcriptional arrangement, transcription from the fHbp promoter itself appeared to be relatively unimportant. The only cc32 isolate in this study that did not transcribe the IG region, NG 080, also had low levels of fHbp transcription when compared with other isolates analysed. Furthermore, there was a good correlation between transcription levels of \textit{cbbA} and fHbp when isolates were grown under iron-replete or -depleted conditions. This is consistent with earlier observations that removal of \textit{cbbA} transcription in MC58 results in significantly reduced levels of fHbp transcription and protein expression (Oriente \textit{et al.}, 2010).

It has been shown that transcription of \textit{cbbA} is dependent on oxygen but independent of FNR. In contrast to the FNR-regulated fHbp, oxygen enhances the rate of transcription of \textit{cbbA} (Oriente \textit{et al.}, 2010). Consequently, under conditions of excess iron and excess oxygen, opposing regulatory mechanisms would be acting on transcription of both \textit{cbbA} and fHbp. Iron and oxygen availability are intrinsically linked \textit{in vivo}, as oxygen potential affects the strength of the association between iron and host iron-binding proteins (Bullen \textit{et al.}, 1992). Iron is also known to be involved in the oxygen-dependent regulatory mechanism of FNR, as the presence of an iron–sulfur cluster within an FNR dimer is required for the interaction with DNA. In the presence of oxygen, this iron–sulfur cluster is displaced (Crack \textit{et al.}, 2004). The relative strengths of iron- and oxygen-dependent regulation on both fHbp and \textit{cbbA} warrant further investigation.

Finally, the observation of an AT-rich IE downstream of the predicted fHbp promoter region in the meningococcal isolate Z2491 has been extended. Comparison of the genome sequences of the 107 reference isolates identified the 181 bp IE at the same location in all ST1, ST4 and ST5 isolates, as well as its presence in alternative positions at least once in all 107 genome sequences. A \textit{BLAST} search of this sequence demonstrated the presence of identical sequence in genomes of \textit{Neisseria gonorrhoeae} isolates. The presence of multiple copies of identical sequence in non-coding regions of the meningococcal genomes is consistent with the hypothesis that this sequence is likely to be an IE (Oriente \textit{et al.}, 2010); however, there is no significant homology with any sequence outside of these species and the sequence does not contain the DNA uptake sequence (Ambur \textit{et al.}, 2007) that is normally associated with DNA IEs in \textit{Neisseria} species. The presence of the IE upstream of the fHbp gene was associated with higher levels of gene transcription following growth in iron-replete media. The mechanism behind this difference is unknown.
but this observation further highlights the complicated nature of transcriptional regulation of the fHbp gene and how it can vary among different meningococcal genotypes.

The results of the present study, combined with earlier observations (Bartolini et al., 2006; Oriente et al., 2010), suggest that fHbp transcription is regulated by both oxygen and iron availability. Assuming that levels of transcription reflect the levels of fHbp expression, this has implications for the use of biological assays, such as the SBA and MATS, in the evaluation of vaccine potency and coverage. For example, SBAs are usually carried out following growth of target isolates on the iron-rich medium blood agar (Maslanka et al., 1997). These conditions enhance transcription of fHbp in most meningococcal isolates, while repressing transcription of fHbp specifically in target isolates belonging to cc32. To offer protection in vivo, antibodies would be required to bind to meningococci in an iron-deficient milieu (Perkins-Balding et al., 2004). Therefore, the in vitro conditions used for SBAs may overestimate the protection offered by vaccine candidates for the majority of meningococcal isolates, while specifically underestimating protection against cc32 isolates, many of which express the fHbp variant 1.1, which is the variant included in the Novartis vaccine formulation (Jacobsson et al., 2009).

Given the close relationship between the regulation of gene expression and the function of the corresponding gene product, the iron-dependent expression of fHbp also raises questions about the predicted role of this protein in meningococcal survival. Increased sensitivity to killing in whole human blood and human sera has been demonstrated in fHbp-knockout mutants (Seib et al., 2010). However, much of this work has been carried out in isolates MC58 and H44/76, both of which are members of cc32. The varying regulation of fHbp in isolates from other ccs, seen in this study, suggests that the ability to bind fH varies widely across different fHbp variants (Seib et al., 2011) and that, in certain isolates, fHbp expression can be removed without any effect on the serum resistance of those isolates (Welsch et al., 2008).

The increasing evidence of an association between antigen expression levels and bactericidal killing (Donnelly et al., 2010) highlights the importance of understanding antigen regulation at a genetic level. Meningococci are known to be highly diverse and adaptable organisms (Feavers, 2000), and it is perhaps unsurprising that regulation of certain ORFs differs among isolates. It is important that this diversity in antigen regulation is investigated and ultimately incorporated into estimates of potential vaccine coverage.

References

Abe, H. & Alba, H. (1996). Differential contributions of two elements of rhd-independent terminator to transcription termination and mRNA stabilization. Biochimie 78, 1035–1042.

Ambur, O. H., Frye, S. A. & Tenjum, T. (2007). New functional identity for the DNA uptake sequence in transformation and its presence in transcriptional terminators. J Bacteriol 189, 2077–2085.

Bartolini, E., Frigimelica, E., Giovinazzi, S., Galli, G., Shaik, Y., Genco, C., Welsch, J. A., Granoff, D. M., Grandi, G. & Grintanti, R. (2006). Role of FNR and FNR-regulated, sugar fermentation genes in Neisseria meningitidis infection. Mol Microbiol 60, 963–972.

Borrow, R., Carlone, G. M., Rosenstein, N., Blake, M., Feavers, I., Martin, D., Zollinger, W., Robbins, J., Aaberge, I. & other authors (2006). Neisseria meningitidis group B correlates of protection and assay standardization–international meeting report Emory University, Atlanta, Georgia, United States, 16–17 March 2005. Vaccine 24, 5093–5107.

Bullen, J. J., Spalding, P. B., Ward, C. G. & Rogers, H. J. (1992). The role of Eh, pH and iron in the bactericidal power of human plasma. FEMS Microbiol Lett 94, 47–52.

Cantini, F., Savino, S., Scarselli, M., Masignani, V., Pizza, M., Romagnoli, G., Swennen, E., Veggi, D., Benci, L. & Rappuoli, R. (2006). Solution structure of the immunodominant domain of protective antigen GNA1870 of Neisseria meningitidis. J Biol Chem 281, 7220–7227.

Crack, J., Green, J. & Thomp­son, A. J. (2004). Mechanism of oxygen sensing by the bacterial transcription factor fumarate-nitrate reduction (FNR). J Biol Chem 279, 9278–9286.

Didelot, X., Urwin, R., Maiden, M. C. J. & Falush, D. (2009). Genealogical typing of Neisseria meningitidis. Microbiology 155, 3176–3186.

Donnelly, J., Medini, D., Boccadifou­co, G., Biolchi, A., Ward, J., Frasch, C., Moxon, E. R., Stella, M., Comanducci, M. & other authors (2010). Qualitative and quantitative assessment of meningococcal antigens to evaluate the potential strain coverage of protein-based vaccines. Proc Natl Acad Sci U S A 107, 19490–19495.

Edwards, J., Cole, L. J., Green, J. B., Thomson, M. J., Wood, A. J., Whittingham, J. L. & Moir, J. W. B. (2010). Binding to DNA protects Neisseria meningitidis fumarate and nitrate reductase regulator (FNR) from oxygen. J Biol Chem 285, 1105–1112.

Feavers, I. M. (2000). Bacterial genomics: ABC of meningococcal diversity. Nature 404, 451–452.

Findlow, J., Borrow, R., Snape, M. D., Dawson, T., Holland, A., John, T. M., Evans, A., Telford, K.-L., Ypma, E. & other authors (2010). Multicenter, open-label, randomized phase II controlled trial of an investigational recombinant meningococcal serogroup B vaccine with and without outer membrane vesicles, administered in infancy. Clin Infect Dis 51, 1127–1137.

Genco, C. A. & Desai, P. J. (1996). Iron acquisition in the pathogenic Neisseria. Trends Microbiol 4, 179–184.
Masignani, V., Comanducci, M., Giuliani, M. M., Bambini, S., Adubobie, J., Arico, B., Brunelli, B., Pieri, A., Santini, L. & other authors (2003). Vaccination against Neisseria meningitidis using three variants of the lipoprotein GNA1870. J Exp Med 197, 789–799.

Maslanka, S. E., Gheesling, L. L., Libutti, D. E., Donaldson, K. B. J., Harakeh, H. S., Dykes, J. K., Arhin, F. F., Devi, S. J. N., Frasch, C. E. & other authors (1997). Standardization and a multilaboratory comparison of Neisseria meningitidis serogroup A and C serum bactericidal assays. Clin Diag Lab Immunol 4, 156–167.

Oriente, F., Scarlato, V. & Delany, I. (2010). Expression of factor H binding protein of meningococcus responds to oxygen limitation through a dedicated FNR-regulated promoter. J Bacteriol 192, 691–701.

Perkins-Balding, D., Ratliff-Griffin, M. & Stojilkovic, I. (2004). Iron transport systems in Neisseria meningitidis. Microbiol Mol Biol Rev 68, 154–171.

Rappuoli, R. (2010). Meningococcal Biology and Vaccines: ten Years of Genomics and Reverse Vaccinology. Presented at 17th International Pathogenic Neisseria Conference, 11–16 September, 2010, Banff, AB, Canada.

Seib, K. L., Serruto, D., Oriente, F., Delany, I., Adu-Bobie, J., Veggi, D., Arico, B., Rappuoli, R. & Pizza, M. (2008). Factor H-binding protein is important for meningococcal survival in human whole blood and serum and in the presence of the antimicrobial peptide LL-37. Infect Immun 77, 292–299.

Seib, K. L., Oriente, F., Adu-Bobie, J., Montanari, P., Ferlicca, F., Giuliani, M. M., Rappuoli, R., Pizza, M. & Delany, I. (2010). Influence of serogroup B meningococcal vaccine antigens on growth and survival of the meningococcus in vitro and in vivo and in vivo models of infection. Vaccine 28, 2416–2427.

Seib, K. L., Brunelli, B., Brogioni, B., Palumbo, E., Bambini, S., Muzzi, A., DiMarcello, F., Marchi, S., van der Ende, A. & other authors (2011). Characterization of diverse subvariants of the meningococcal factor H (Hf) binding protein for their ability to bind FH, to mediate serum resistance, and to induce bactericidal antibodies. Infect Immun 79, 970–981.

Snape, M. D. & Pollard, A. J. (2005). Meningococcal polysaccharide-protein conjugate vaccines. Lancet Infect Dis 5, 21–30.

Welsch, J. A., Ram, S., Koeberling, O. & Granoff, D. M. (2008). Complement-dependent synergistic bactericidal activity of antibodies against factor H-binding protein, a sparsely distributed meningococcal vaccine antigen. J Infect Dis 197, 1053–1061.

Yongye, A. B., Gonzalez-Outeiriño, J., Glushka, J., Schultheiss, V. & Woods, R. J. (2008). The conformational properties of methyl z-(2,8)-di/trisialosides and their N-acyl analogues: implications for anti-Neisseria meningitidis B vaccine design. Biochemistry 47, 12493–12514.

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