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Investigating the regulation of the vaccine antigen Factor H binding protein in *Neisseria meningitidis*

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1. Abstract

Neisseria meningitidis is a strictly human pathogen and is a major cause of septicemia and meningitis worldwide. Factor H binding protein (fHbp) is a surface-exposed lipoprotein that binds human factor H, a negative regulator of the complement cascade, allowing the bacterium to evade the host innate immunity response. Of note, fHbp is a key antigen in two novel vaccines against *N. meningitidis* serogroup B (Bexsero® and Trumenba®). Although the *fHbp* gene is present in most circulating meningococcal strains, the level of fHbp expression varies among isolates and may influence strain susceptibility to anti-fHbp antisera. The aim of the PhD project was to understand the sequence determinants that control fHbp expression in globally circulating strains. We analyzed the upstream *fHbp* intergenic region (fIR) of a panel of 105 invasive strains representative of the epidemiology of the three fHbp variants and we identified nine fIR sequence types which represent 77% of the isolates. By quantitative selected reaction monitoring mass spectrometry we obtained an absolute quantification of fHbp in the same panel of strains and found a correlation between the fIR sequence type and fHbp expression levels. By the generation of a series of isogenic recombinant strains, where fHbp expression was under the control of each of the nine fIR types, we were able to confirm that the fIR sequence determines a specific level of expression, both at the mRNA and the protein level. Moreover, the molecular bases for variation in expression through SNPs within key regulatory regions that affect fHbp expression were identified. Furthermore, our data indicate an influence of the variant coding sequence in the stability of the protein, hence suggesting a regulation of the antigen at transcriptional and post-transcriptional levels. In order to investigate the regulatory effect of different stimuli encountered by *Neisseria meningitidis*, we compared transcript levels in the isogenic recombinant strains in the presence or absence of either oxygen or iron. Our data showed an increase of *fHbp* transcripts under oxygen-limiting conditions for most of the mutant strains and a general downregulation of the gene upon iron-depletion. In addition, the quantity of fHbp on the surface of the bacteria correlated directly with the susceptibility to killing mediated by anti-fHbp antibodies in immune sera. The influence of fHbp to mediate the evasion from generic complement-mediated killing presumably through binding of human fH was assessed and survival in human non-immune

serum was less correlated with protein amounts measured from an *in vitro* growth culture. These results suggest a possible regulation of *fHbp* in the presence of human serum for at least some of the fIR types under investigation. Finally, an extensive analysis done on the *fHbp* intergenic region of more than 900 strains representative of the UK circulating isolates identified 11 fIR sequence types which represent 89% of meningococcal strains. Moreover, among them only two new fIR types were identified. Statistical analyses suggested the evolution of the *fHbp* intergenic region with its corresponding coding sequence, with the peculiar exception of strains harboring var2 which have evolved to maintain the same regulatory elements for the tuning of fHbp expression. Overall, we demonstrated that the expression level of this important antigen can be inferred by the DNA sequence of the *fHbp* intergenic region. Therefore, our findings can contribute to understand and predict vaccine coverage mediated by fHbp.

2. Introduction

2.1 Meningococcal disease

Neisseria meningitidis is an exclusively human pathogen, which is responsible for meningitis and sepsis, two devastating diseases that can kill children and young adults within hours, despite the availability of effective antibiotics. The first description of a meningococcal infection of the cerebrospinal fluid of a patient was done by Anton Weichselbaum in 1887 [1]. Studies performed in Europe have demonstrated that carriage rates are very low in the first few years of life, but rise during adolescence, reaching peaks of 10-35% in 20-24 years old people, and decreasing to less than 10% in older groups [2, 3]. With the respect of carriage rates, meningococcal disease is rare, varying from 0.5 to 10 per 100,000 persons; however, the incidence can rise above 1 per 1,000 during epidemics [4, 5]. Approximately 5 to 15% of people contracting meningococcal disease dies, and the rate increases to 40-55% in case of sepsis [6, 7]. Furthermore, from 11 to 19% of individuals surviving the disease often suffer from permanent sequelae, including hearing loss, seizures, neurodevelopmental deficits, ataxia, hemiplegia, as well as amputation of limbs [6-10]. The factors determining the transition from colonization to disease state are not fully understood. However, certain biological, environmental and social factors have been associated with an increased risk of disease in otherwise healthy individuals. Infants under one year of age have the highest risk of infection due to their immature immune systems (6.33-7.08 cases per 100,000). Whereas, the peak observed in adolescents is largely due to increased carriage in this population [11]. Several studies have demonstrated that factors both at the pathogen and at the host levels influence disease development, such as microbial virulence factors, human genetic polymorphisms, impaired immune system, as well as environmental conditions facilitating exposure and acquisition, and naso- and oro-pharyngeal irritation caused by smoking and respiratory tract infection [6, 12-17].

The diagnosis of meningococcal disease can be arduous since its symptoms, like headache, fever and rash, are unspecific, especially in the onset and early stages of the infection, and may be confused with other less life-threatening pathologies. Because of its rapid progression, any delay

in antibiotic treatment can lead to death within 24 to 48 hours from the first manifestations [10]. Hence, vaccination represents the unique effective public health response.

2.2 *Neisseria meningitidis*: pathogen and pathogenesis

Neisseria meningitidis is a Gram-negative β-proteobacterium member of the Neisseriaceae family. It is an aerobic, non-motile and non-sporulating diplococcus (Figure 1), usually encapsulated and piliated. The envelope of *N. meningitidis* consists of the cytoplasmic membrane, the outer membrane (OM) and the periplasm between them, which contains a layer of peptidoglycan. The cytoplasmic membrane is a phospholipid bilayer, whereas the OM is composed of a phospholipidic inner leaflet and an outer leaflet of lipooligosaccharide (LOS). Some meningococcal strains have a polysaccharide capsule attached to their OM and almost all pathogenic strains are encapsulated. Nevertheless, also non-encapsulated isolates have been recently associated to invasive disease [18].

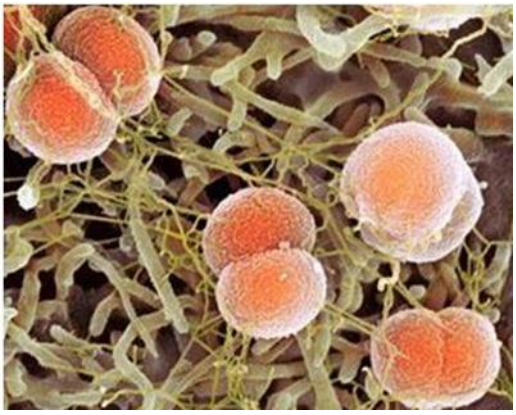

Figure 1. *Neisseria meningitidis* diplococci. Colored scanning electron micrograph of *Neisseria meningitidis* bacteria on human epithelium (modified from www.sciencephoto.com).

On the basis of the bacterial polysaccharide capsule, it is possible to classify meningococcus into 13 serogroups, six of which, A, B, C, Y, X and W135, are associated with the majority of the disease worldwide [4, 19-21]. Meningococci are further classified into serotype and serosubtype according to antigenic differences in their major outer membrane proteins, PorA and PorB. However, the classification based on the serological characteristics of *N. meningitidis* is limited

due to the high frequency of variation of OM-proteins, probably determined by a strong selective pressure. Hence, new DNA-based methods for the characterization of meningococcal isolates have been developed, and the Multi Locus Sequence Typing (MLST) is now considered the gold standard for molecular typing and epidemiologic studies [22]. This typing system relies on polymorphisms within seven housekeeping genes; each sequence for a given locus is screened for identity with already known sequences for that locus. If the sequence is different, it is considered to be a new allele and is assigned an identification number. Therefore, the combination of the seven allele numbers determines the allelic profile of the strain, and each different allelic profile is assigned as a sequence type (ST). Meningococci sharing at least four of the seven loci with a central ancestral genotype are grouped together into clonal complexes (CCs) [23]. Through the employment of MLST it has been shown that the majority of strains associated with invasive disease belong to specific CCs (ST-1, ST-4, ST-5, ST-8, ST-11, ST-32, ST41/44 and ST-269), called hyper-invasive [24, 25]. However, the reasons of this enhanced pathogenic phenotype are yet unknown.

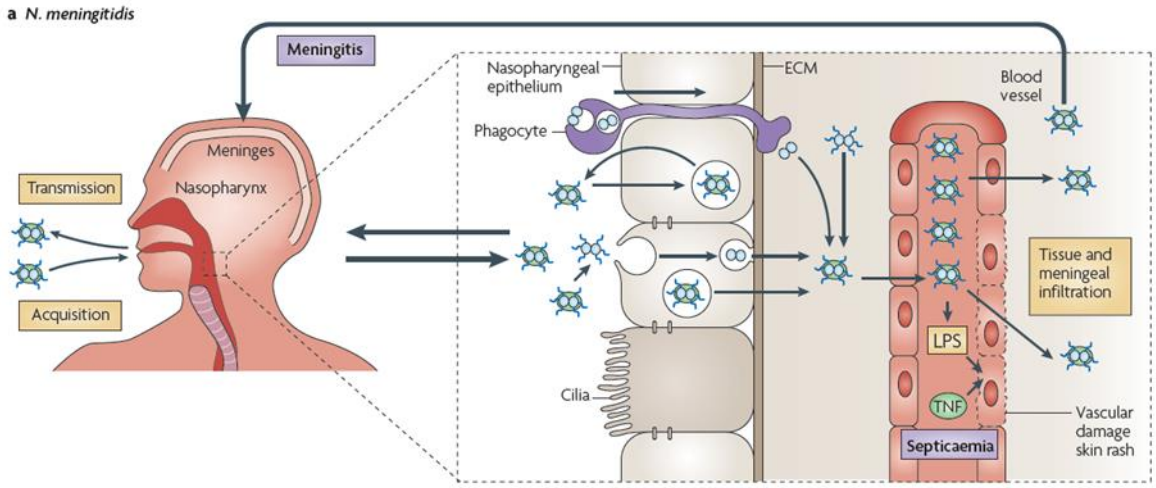

Figure 2. Stages in the *Neisseria meningitidis* pathogenesis. Representation of the steps that characterize the meningococcal colonization and infection (adapted from [26]).

The pathogenesis of *Neisseria meningitidis* is a complex multi-stage process (Figure 2). Meningococci might be transmitted via respiratory droplets or saliva and colonize the upper respiratory tract of the human host. The first colonization step is conserved by both the carriage

state and the invasive disease [27] and consists in the initial contact with nasopharyngeal epithelial cells mediated by Type IV pili, which may recognize the host receptor CD46 [28]. Then, bacteria proliferate on the surface of human non-ciliated epithelial cells, forming small micro-colonies at the site of initial attachment [27]. After this step, the capsule, which masks the OM proteins via steric hindrance, is lost or down-regulated due to cell-contact induced repression [29] or selection of low or no-capsule expressing bacteria caused by phase variation [30]. The absence of the capsule reveals a variety of redundant adhesins, which mediate a close adherence of the bacteria to host epithelial cells [27]. The interaction of bacterial opacity proteins, Opa and Opc, with CD66/CEACAMs and integrins, respectively, on the surface of epithelial cells triggers meningococcal internalization [31]. This causes the appearance of cortical plaques and the recruitment of factors leading to the formation and extension of epithelial cell pseudopodia that encircle bacteria within intracellular vacuoles [32]. The survival of internalized meningococcal cells is dependent on their capability to evade the immune response and to acquire nutrients. Indeed, iron acquisition mediated by specialized transport systems, such as the transferring binding protein (TbpAB), the lactoferrin binding protein (LbpAB), and the hemoglobin binding receptor (HmbR) is partially responsible for meningococcal intracellular replication [33]. In healthy individuals, bacteria that cross the mucosal epithelium are eliminated by serum bactericidal activity. Nonetheless, on occasion bacteria can cross the mucosal epithelial barrier of susceptible individuals, either through transcytosis or through phagocytes, or directly following damage to the monolayer integrity [26], and eventually enter the bloodstream. Survival within human blood relies upon different mechanisms. The up-regulation of capsule expression prevents the deposition of both antibodies and complement factors [34] hence inhibiting phagocytosis. Other strategies developed by the bacteria to evade the immune system are the recruitment of negative regulators of the complement cascades, such as Factor H (fH), which is bound by the Factor H binding protein (fHbp) [35], or by the Neisserial surface protein A (NspA) [36], and by the Porin B (PorB) [37], or the recruitment of complement regulators, such as the C4-binding protein, which is bound by Porin A (PorA) [38]. Once inside the bloodstream, meningococci can multiply slowly and eventually cross the blood-brain barrier, causing the infection of meninges and cerebrospinal fluid [39]. Otherwise, in case of rapid multiplication within the blood, the bacteria cause septicemia or meningococcemia [6, 40].

From an evolutionary point of view the onset of the meningococcal disease represents a failed or dysfunctional relationship between the bacteria and the host, since eventually their fates are interconnected.

2.3 Virulence factors

The virulence of *N. meningitidis* is influenced by multiple factors that are mainly located in the OM (Figure 3). The main virulence factor is the polysaccharide capsule which protects the bacterium from desiccation during airborne transmission between hosts [41], and protects it from the host innate and adaptive immune system [42, 43]. Its expression is phase variable [30] and the switching of the capsule locus between strains confers a selective advantage to the bacterium for its evasion to opsonization or neutralization by natural or vaccine-induced anti-capsular antibodies [44].

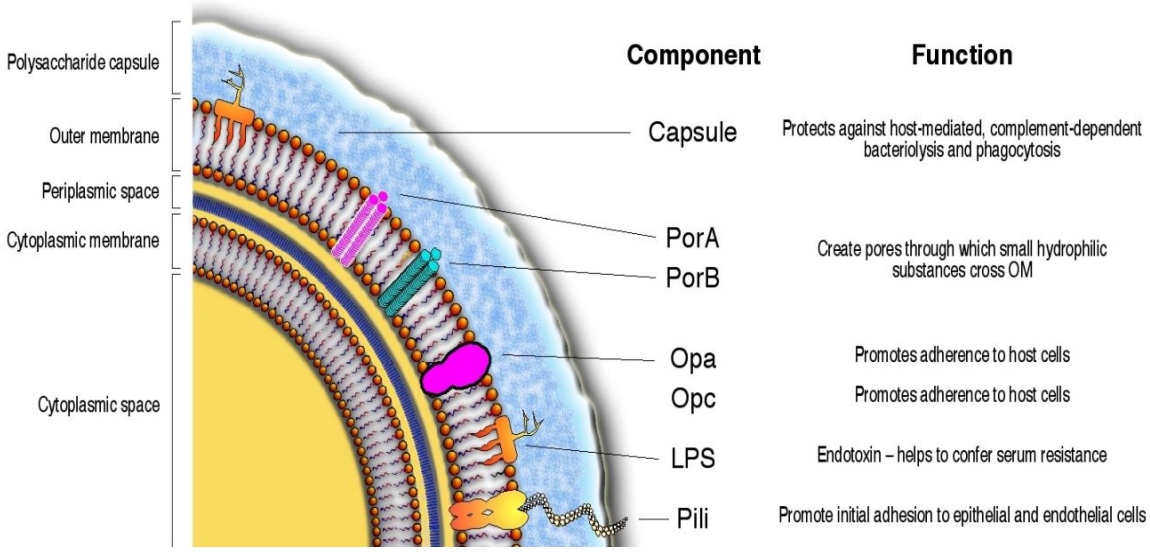

Figure 3. Meningococcal cell compartments. Representation of the different bacterial compartments and of the main components of the outer membrane, together with their known function [6].

The meningococcal LOS is composed of short saccharides and is responsible for the physical integrity and proper functioning of the membrane and required for resistance of *N. meningitidis* to complement [45]. Phase and antigenic variations lead to different saccharide chains altering

dramatically the antigenic properties of LOS and enabling individual meningococci to display a repertoire of multiple LOS structures simultaneously [46].

Another group of virulence factors involved in the interface between the meningococcus and the host are pili. They are long filamentous structures consisting of protein subunits that extend from the bacterial surface beyond the capsule [47, 48]. Pili represent the major contributor to the adhesive property of the capsule [49, 50] and in addition they are involved in the uptake of foreign DNA from the extracellular environment, hence increasing transformation frequency and consequently genetic adaptability [51].

Furthermore, the presence of other OM-associated proteins is important in host cell interaction. The opacity proteins (Opa and Opc) are integral outer membrane proteins that mediate pathogen-host interaction, adhering to and invading of epithelial and endothelial cells [48]. A key role in the adhesion is carried out by adhesins, which are generally low expressed *in vitro*, but they might be upregulated *in vivo*. In fact, they may undergo to antigenic variation and/or phase variation, hence allowing the meningococcus to evade the immune system and adapt to different niches [26]. The Neisserial adhesin A (NadA) is a surface-exposed member of the Oligomeric coiled-coil adhesin family of bacterial Trimeric Autotransporter adhesins [52, 53]. NadA mediates adhesion to and invasion of human epithelial cells [54], suggesting its pivotal role in the adhesion to the naso- and oro-pharyngeal epithelia during meningococcal colonization of the human upper respiratory tract. Other adhesins have been reported to play a role in colonization and/or invasion. The Meningococcal surface fibril (Msf), previously termed Neisseria hia homologue A (NhhA) and *H. influenzae* surface fibril (Hsf) [55, 56], mediates adhesion to epithelial cells and to components of the extracellular matrix, even though at low levels [57]. Moreover, it has been shown its involvement in the immune system evasion. Msf binds to the activated form of Vitronectin and inhibits the terminal complement pathway [58], and its role in inhibiting phagocytosis, inducing macrophages apoptosis and protecting bacteria against complement-mediated killing has been suggested [59, 60]. Two homologous autotransporters, the Adhesion penetration protein (App) and the Meningococcal serine protease A (MspA) are involved in the bacterial interaction to epithelial cells [61, 62] and also in the apoptosis of dendritic cells [63]. Glycolipid adhesins such as members of the Multiple adhesin family (Maf) may contribute to the bacterial interaction with host cells [64]. Interestingly they are found to be

associated with genomic islands present only in pathogenic *Neisseria* species, both meningococcus and gonococcus [65].

The two porins PorA and PorB, are the most abundant proteins present in the meningococcal OM. They are composed of relatively conserved regions, which are predicted to form the β-barrel structure that spans through the membrane, alternated with variable regions, which should be surface-exposed, hence undergoing to a strong selective pressure. The formation of trimers creates the pore structure that allows the passage of small hydrophilic solutes necessary for the bacterial metabolism. Porins were shown to be interacting with several human cell types and proteins [66]; moreover, PorA elicits a protective immune response in humans [67, 68], while PorB might be involved in the immune system evasion by binding the human fH (hfH) [37].

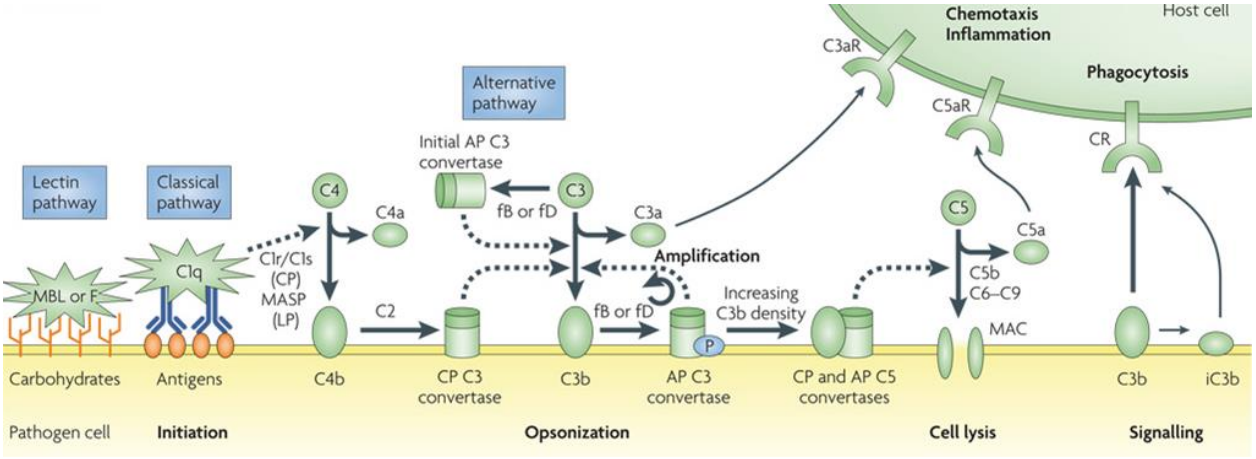

Figure 4. Activation of complement. Schematic representation of the three pathways (Lectin, Classical and Alternative Pathway) that trigger the activation of the complement system and its following steps leading to the killing of the pathogen.

Furthermore, the genome of *N. meningitidis* contains a set of membrane-associated factors responsible for the host's immune system evasion and hence for its virulence. As indicated by the elevated susceptibility to microbial, including meningococcal, infections exhibited by individuals with complement deficiencies [69], the complement system is pivotal in the defense against *N. meningitidis* infection. This essential component of the innate immune response is composed of a network of cell surface-associated and circulating proteins that act as modulators, substrates or enzymes of a hierarchical and finely-tuned series of extracellular proteolytic cascades. Three

pathways lead to the activation of the complement system (Figure 4). The classical pathway (CP) is triggered by the binding of the hexameric C1q to antigen-antibody complexes on foreign cell-surfaces; whereas, the lectin pathway (LP) is stimulated by the recognition of carbohydrate ligands present on the microbial surface. On the other hand, the presence of external surface structures or the spontaneous hydrolysis of C3 initiate the alternative pathway (AP). All of these three pathways converge in the cleavage of the C3 by the C3 convertase, which leads to the formation of the active fragments C3a and C3b. The deposition of C3b on the bacterial surface induces the phagocytosis of the foreign cell or its lysis through the activation of the C5 convertase and the cleavage of C5, which in turn leads to the formation of a multiprotein pore complex, the membrane attack complex (MAC). Activation and repression of the complement system must be strictly regulated in terms of both space and time. Therefore, the presence of several regulators, such as factor H, factor H-like protein-1 (FHL-1) and C4-binding protein (C4BP), allows complement physiological function. In order to escape from the innate immune system, *N. meningitidis* has evolved a plethora of mechanisms that target the complement cascades. As already introduced above, at least three meningococcal proteins are shown to bind the fH, fHbp [35], NspA [36] and PorB [37]. fHbp will be discussed below in details. NspA was firstly identified in a fHbp deleted background, for its ability to bind hfH. Strains lacking both fHbp and NspA were not able to bind fH and indeed were more susceptible to complement-dependent killing [36, 70]. In addition, the observed binding of heparin from the Neisserial Heparin-Binding Antigen (NHBA) may increase bacterial serum resistance due to the potential interactions of heparin with fH [71].

2.4 Factor H binding protein (fHbp)

Factor H binding protein is a 27 kDa surface-exposed lipoprotein, previously referred as GNA1080 [72] or LP2086 [73], and is an important virulence factor of *N. meningitidis* [74] as it binds the human Factor H [35, 75], hence down-regulating the alternative complement pathway and allowing the meningococcus to evade the immune system [76, 77]. Moreover, it has been shown that fHbp is uniquely able to bind the human fH and not the rat or rabbit fH, hence explaining the species specificity for meningococcal infection [75]. The importance of fHbp for

the immune system evasion was also demonstrated by incubating *fHbp*-deletion mutants in human blood or serum. Following incubation, mutant strains lacking this gene were killed rapidly, whereas wild type and complemented strains survived [70, 78, 79]. Moreover, fHbp was found to confer protection against the antimicrobial peptide LL-37 [78].

Its three-dimensional structure, determined by both nuclear magnetic resonance (NMR) [80] and X-ray crystallography [81], reveals that the protein is composed of two independent β-barrel domains connected by a short linker (Figure 5A). fHbp is expressed as a precursor containing the lipoprotein signal motif (LXXC), which is necessary for the correct targeting of the protein [72, 82]. In the periplasmic site of the inner membrane the precursor is cleaved just upstream of the cysteine present within the signal motif and the protein is anchored to the membrane through the lipidation of the cysteine. The lipoprotein is then translocated to the OM and flipped via an unknown mechanism to the outer leaflet of the OM [82].

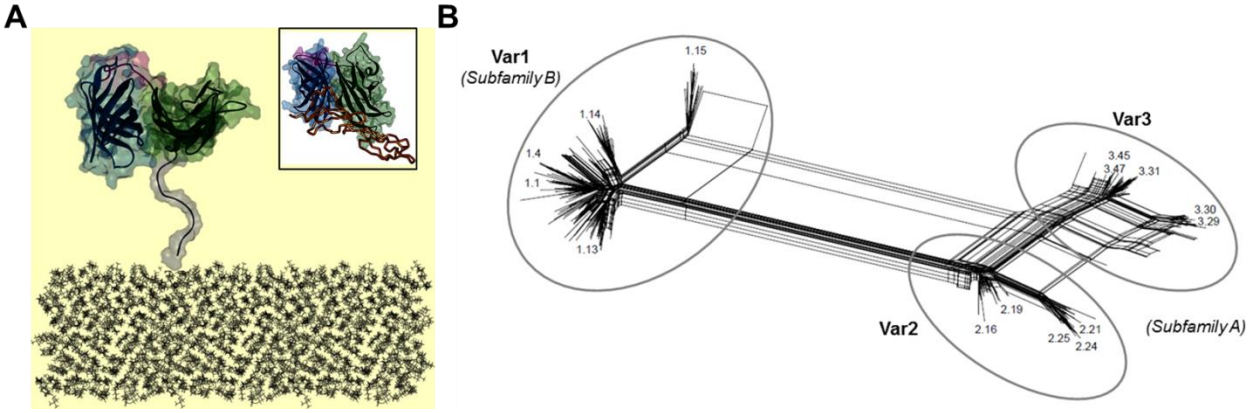

Figure 5. Factor H binding protein. (**A**) Schematic representation of fHbp anchored on the surface of *N. meningitidis*. The fHbp structure is colored as follows: white, N-terminal flexible stem; green, N-terminal domain; blue, C-terminal domain; pink, linker between the β-structures of the two domains. In the inset, is represented the structure of a molecule of fHbp binding the human factor H (in orange) (adapted from [83]). (**B**) Phylogenetic distribution of fHbp by SplitsTree analysis of 258 different sub-variants present. The tree shows the clustering of the proteins in the three main variants (Var1, Var2 and Var3) and in the two subfamilies (A and B). The 5 most representative sub-variants for each variant are indicated. Length of the lines indicates genetic distance.

The sequence of the *fHbp* gene in more than 7,000 clinical isolates currently present in public databases identified more than 860 different alleles, or peptides, of the protein that have been divided into three main variants (var1, var2, and var3) or two subfamilies (A and B, corresponding to variants 2/3 and 1, respectively) [72, 73], each of which can be further

distinguished in many peptide subvariants or subfamily groups (http://pubmlst.org/neisseria/fHbp/) (Figure 5B). In a global population of MenB the variants 1, 2 and 3 are present in approximately 65, 25 and 10% of the isolates, respectively [84-87]. It has been demonstrated that fHbp proteins from different variant groups do not induce cross-protection; in details, the antibodies raised against one fHbp subvariant are not able to protect against strains harboring a subvariant from a different variant group [72, 73].

Interestingly, the *fHbp* gene is also present in other species of the genus *Neisseria*, both commensals, *N. cinerea* (*fHbp* var1) and *N. polysaccharea* (often frame-shifted *fHbp* var3), and pathogenic, *N. gonorrhoeae* (fHbp var3) [88]. The gonococcal fHbp (Ghbp) is not able to bind the hfH and is not surface exposed due to the presence of a frameshift within the N-terminal region that impairs the lipoprotein signal motif [88, 89].

Although the *fHbp* gene is present in most circulating meningococcal strains, its levels of expression vary significantly between isolates [72, 73, 85, 90]. Moreover, recent studies have identified invasive isolates with frame-shift mutations which abrogate fHbp protein expression [72, 84, 91]. The *fHbp* gene was originally annotated as NMB1870 according to its location within the genome sequence of the strain MC58 [92]. Upstream and in the same orientation is *cbbA*, annotated as NMB1869, coding for a fructose-bisphosphate aldolase [93] (Figure 6). The annotation of *fHbp* is controversial: the start codon as deposited by The Institute for Genomic Research (TIGR) is located 19 bp downstream of the stop codon of the *cbbA* gene [92] (Figure 6, boxed ATG); whereas, the Sanger Centre positioned the start codon 139 bp downstream of it [94] (Figure 6, dash boxed ATG). In a later work, Masignani *et al*. [72] proposed a new annotation for the gene, in which the start codon is the GTG triplet 15 bp downstream the Sanger's ATG (Figure 6, bold GTG). Moreover, a putative ribosome binding site (AGGAG) maps at 7 nt upstream of the GTG triplet (Figure 6, underlined).

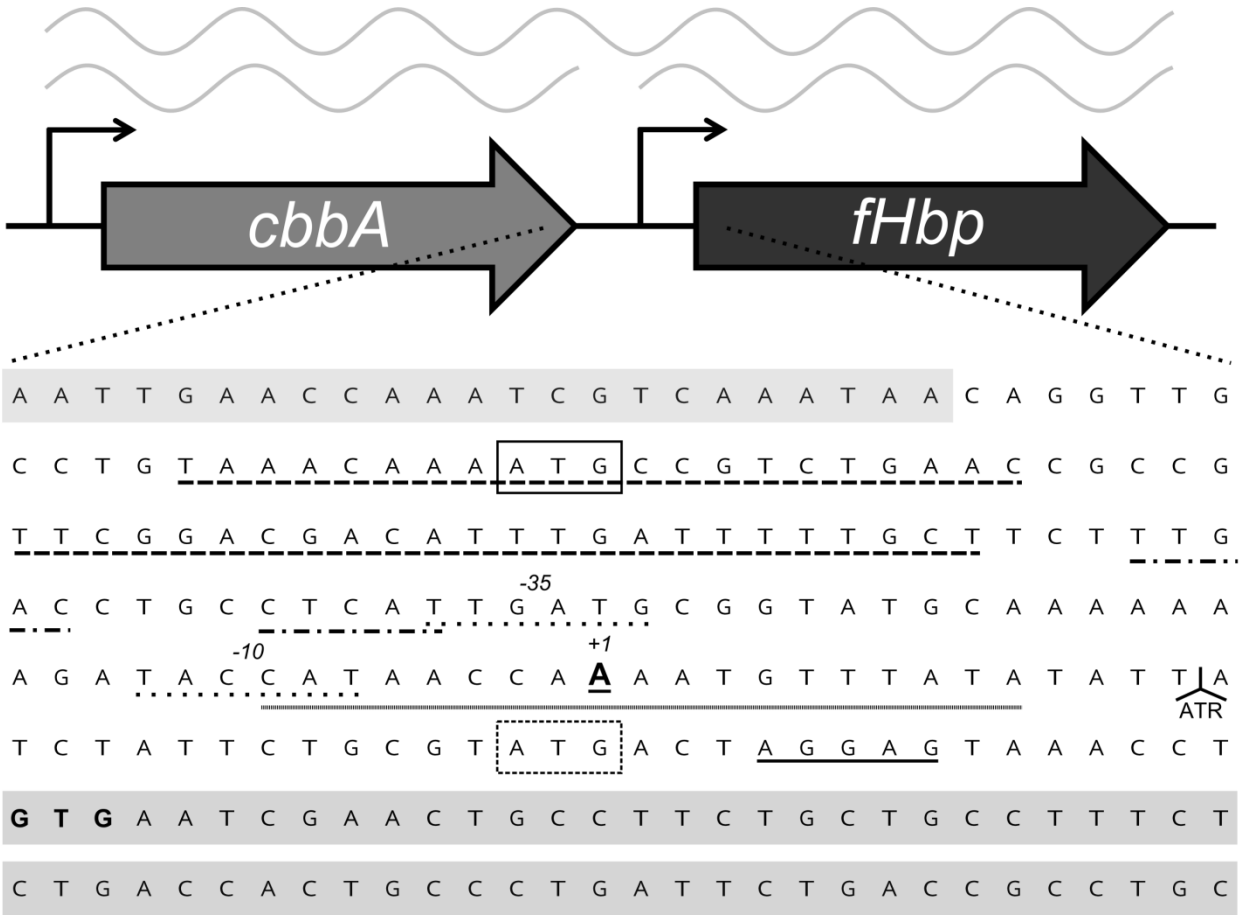

Figure 6. *fHbp* locus in MC58 strain. Schematic representation of the locus of *fHbp* with its upstream *cbbA* gene and the transcripts generated from both promoters (upper panel). In the lower panel is reported the nucleotide sequence of the region under investigation in this study as it is in [72]. The 3' region of *cbbA* is boxed in light grey and the 5' region of *fHbp* is boxed in dark grey. The three proposed translational start sites of *fHbp* are indicated (boxed, dash boxed and in bold). The ribosome binding site AGGAG is underlined; the -35 and -10 elements of the P_{fHbp} are indicated and underlined (dotted line). The transcriptional start site is in bold and underlined. The nucleotides pairing in the stem region of the Rho-independent terminator are underlined (dashed lines) as well as the binding site of the transcriptional factor FNR (dot-and-dash lines) and the putative Fur-box (thin dashed line). The site of insertion for the ATR element is indicated.

Oriente *et al*. demonstrated that in the reference strain MC58 the transcripts of *cbbA* and *fHbp* are originated independently from the P_{cbbA} and P_{fHbp} promoters respectively, and that a bicistronic mRNA can originate from P_{cbbA}, hence adding *fHbp* transcript [90]. The presence of the longer mRNA was predicted to be dependent on the strength of the Rho-independent terminator present within the *cbbA-fHbp* intergenic region (Figure 6, dashed line). Indeed, the analysis of the DNA sequence of this region in other meningococcal strains highlighted the presence of polymorphisms which might impair the correct palindromic stem-loop structure. A weak

terminator, as is it in MC58, would allow the read-through of the RNA polymerase from the upstream promoter, hence resulting in a bicistronic transcript [90, 95]. The -35 (TTGATG) and -10 (TACCAT) elements of the P_{fHbp} promoter were identified (Figure 6, dotted line) and interestingly, mutations in the -10 box (TACCGC) were associated with a low fHbp expression in the strain NM117 [90]. The analysis of a global panel of meningococcal isolates led to the identification of a subset of strains harboring an insertion element of 187 bp rich in A and T (ATR) downstream of the mapped *fHbp* promoter [72, 90, 95] (Figure 6). This sequence may affect fHbp expression in these strains either transcriptionally (by containing promoter or terminator regulatory sequences) or post-transcriptionally, as it will be transcribed in both *fHbp* transcripts and may affect mRNA stability [90].

During infection, *N. meningitidis* can invade diverse sites within the human host, hence is subjected to different environmental conditions and its ability to rapidly adapt its metabolism and cellular composition is essential for its survival [96].

The availability of iron varies within the human host and the presence of a putative Fur-box motif within the intergenic region of *fHbp* suggests an iron regulation of the gene when the bacteria encounter different niches [72] (Figure 6, thin dashed line). Indeed, Sanders *et al*. demonstrated that *fHbp* transcription is regulated by iron availability; in details, the majority of the strains expressed significantly less levels of *fHbp* when grown under iron depletion, while strains belonging to the CC32 were upregulated. Hence, they suggested that iron regulation is dependent on the genetic background of the strain, specifically the clonal complex [95]. The presence of the ATR-insertion element was associated with higher levels of *fHbp* transcription following growth in iron-replete media [95].

One of the features of the inflammation process is the increase in temperature. The upregulation of factors involved in the immune evasion under these conditions (thermoregulation) would provide an advantage when the meningococcus passes from the nasopharynx (32-36°C) to the bloodstream (37°C) [97]. Loh *et al*. showed that the increase in temperature determines an increase in the fHbp protein amounts [98]. Secondary structures of the mRNA, thermosensors, would account for the thermoregulation, facilitating the access of the ribosome to the RBS at higher temperatures. The region proposed to be involved in this mechanism was the 5'UTR plus part of the coding sequence of *fHbp* (27 nt from the GTG start codon) (Figure 6) [98].

N. meningitidis would be exposed to highly different partial pressures of oxygen as the bacteria move from the surfaces of the upper respiratory tract (21 kPa) to mucus membranes (0.4 kPa buccal fold pressure), blood (4 kPa central venous pressure), and cerebrospinal fluid (5 kPa) [99]. Under oxygen limitation meningococcus uses the fumarate and nitrate reductase (FNR) global transcription factor to induce sugar fermentation and denitrification pathways, utilizing nitrite and nitric oxide as electron acceptors [100, 101]. In the absence of oxygen FNR binds to DNA and activates target genes as a dimer containing a [4Fe-4S] cluster. This cluster dissociates in the presence of oxygen, destabilizing the dimer, with loss of FNR activity [101, 102]. Among the genes regulated by FNR there is *fHbp* [101]. The *cbbA-fHbp* intergenic region contains a binding site for FNR (TTGAC-N_4-CTCAT) (Figure 6, dot-and-dash line) and studies demonstrated the FNR-dependent upregulation of *fHbp* in the absence of oxygen [90]. Notably, the response of the P_{fHbp} was FNR-dependent and apparently, the expression of *cbbA* decreased under oxygen limiting conditions, in an FNR-independent manner [90, 95]. Moreover, the FNR-binding site sequence was found to be conserved in the panel of strains analyzed, indicating a conserved oxygen-response regulation among isolates [90]. These evidences suggest that in microenvironments where oxygen is limiting, such as the bloodstream, *N. meningitidis* ensures the expression of fHbp. The findings that fHbp expression is induced during growth in human blood [70, 103] and that antibodies against fHbp can be recovered from sera of convalescent patients and from carriers [104, 105] provide additional evidences supporting the pivotal role of fHbp in invasive meningococcal disease.

2.5 Meningococcal vaccines

Due its rapid progression and the difficulties to diagnose it [6, 10], the most effective option to prevent meningococcal disease is vaccination. No broadly protective vaccine is currently available to provide protection against all serogroups of *N. meningitidis*. Capsular polysaccharides have been successfully used as antigens to produce polysaccharide and glycoconjugate vaccines against serogroups A, C, W135 and Y, and monovalent, bivalent, and quadrivalent vaccines have been developed and licensed [106]. In contrast, the group B capsule polysaccharide is not suitable as vaccine antigen because it consists of a homolinear polymer of

α(2→8)N-acetyl neuraminic acid, which is structurally similar to the sialic acid found in human neural tissue, hence is poorly immunogenic in humans and may elicit auto-antibodies [107, 108]. Therefore, efforts to develop a vaccine against meningococcus serogroup B (MenB) focused mainly on non-capsular antigens, such as LOS or proteins. In order to control outbreaks caused by specific MenB strains vaccines composed of detergent-extracted Outer Membrane Vesicles (dOMV) have been successfully employed in Norway [109], Cuba [110], Chile [111] and New Zealand [112]. The process of detergent extraction removes the toxic LOS, but also other desirable antigens present in the OMVs, such as lipoproteins. Consequently, PorA results to be the immuno-dominant antigen [113, 114]. Because of PorA antigenic variability [115], the immune response elicited is effective only against strains expressing the same PorA serosubtype. Therefore, the breadth of coverage provided by dOMV vaccine is limited. The advent of the genomic era and the availability of whole genome sequences have contributed to radically change the approach to vaccine development. Indeed, the *in silico* approach named Reverse Vaccinology (RV) aims to identify surface-exposed non-capsular antigens that are (i) antigenically conserved among strains and (ii) elicit a bactericidal serum response. RV led to the development of the recombinant protein vaccine Bexsero® (former known as 4CMenB) [116, 117]. This vaccine contains five antigens formulated together with the dOMV component from the NZ98/254 strain [114]. The antigens are NadA [54, 118], and two recombinant fusion proteins of fHbp [72, 119] and NHBA [71, 120] fused to the conserved meningococcal gene products NMB2091 and NMB1030, respectively. NadA, fHbp and NHBA represent the major antigens that were selected through RV, based on their ability to induce broad protection [116]. The other two antigens, NMB2091 and NMB1030, are well conserved in *N. meningitidis*, but less functionally characterized than the other antigens [88, 121, 122]. They were included in the vaccine formulation since they increase immune responses to the main vaccine antigens when present as fusion proteins with the respect of the individual antigens [116]. Bexsero was licensed in Europe in 2013 and in the U.S. in 2015, following its progression through clinical trials that have demonstrated its safety [123-125] and its efficacy in inducing a protective immune response in infants, children, adolescents and adults against the majority of MenB strains [126-132].

The other available vaccine against *N. meningitidis* serogroup B is Trumenba® and it was licensed in the U.S. in 2014 for a target population of adolescents and young adults. It is a

recombinant protein-based vaccine composed of equal amounts of two variants, subfamily A05/var3.45 and subfamily B01/var1.55, of lipidated fHbp [73]. A preclinical study suggested a good breadth of coverage [133] and through a phase I clinical trial was assessed its tolerability in adults, adolescents and young children [133]. However, it is not suitable for use in infants considering that it consists of purified lipoproteins known as TLR-2 agonists [134].

2.6 Vaccine coverage prediction

In 1969 Goldschneider showed a correlation between resistance to meningococcal meningitis and the presence of bactericidal antibodies in the serum [13]. This presence is determined by using the Serum Bactericidal Assay (SBA) in the presence of human complement (hSBA), which is widely accepted as a surrogate marker of protection against meningococcal disease [13, 135]. The employment of hSBA for evaluating vaccine effectiveness to kill meningococcal circulating strains presents some issues, because it requires large amounts of reagents not always of easy access, especially in the case of infant serum, for which only small volumes are available. Moreover, the output of the analysis is due to the cumulative effect of the entire set of antibodies present within a serum sample, hence the individual contribution of each antigen of the vaccine is not easily understandable. This is especially the case of the multicomponent vaccine against *N. meningitidis* serogroup B, Bexsero, for which a novel assay, the Meningococcal Antigen Typing System (MATS), has been developed [136] (Figure 7). This system allows the simultaneous assessment of both the antigenic cross-reactivity and the level of expression of the surface-exposed antigens of an unknown *N. meningitidis* isolates with the respect to reference MenB strains for a specific antigen. Given the high variability of PorA, the sequencing of the *porA* locus and the determination of its subtype are sufficient for determining whether a strain would be killed: only strains carrying the same subtype as the one present in the vaccine, PorA P1.4, are efficiently killed by Bexsero-induced immune sera. MATS evaluates the protection induced by the other recombinant antigens, fHbp, NHBA and NadA, by means of ELISA. A suspension of bacteria prepared from an overnight growth on chocolate agar is treated with detergent to extract the capsule and expose the antigens and is tested in a MATS ELISA plate, one for each recombinant antigen, which is coated with antibodies raised against the specific antigen. Results

of the ELISA are compared to the results obtained for the reference strain and the output of the MATS, the Relative Potency (RP), correlates with the hSBA and predicts if the strain tested would be killed by the antibodies elicited by immunization with Bexsero. For each one of the antigens, RP thresholds values for antibodies-mediated killing of MenB strains were determined (Positive Bactericidal Threshold - PBT -). MATS can be employed for the evaluation of large panel of strains, allowing the determination of potential strain coverage by Bexsero in a target geographic region [137-140]. Coverage prediction by MATS is used to support the licensing of Bexsero around the world. According to MATS, it has been estimated that 78% of circulating MenB strains in five European countries would have at least one antigen rated above the PBT and therefore would be covered by Bexsero.

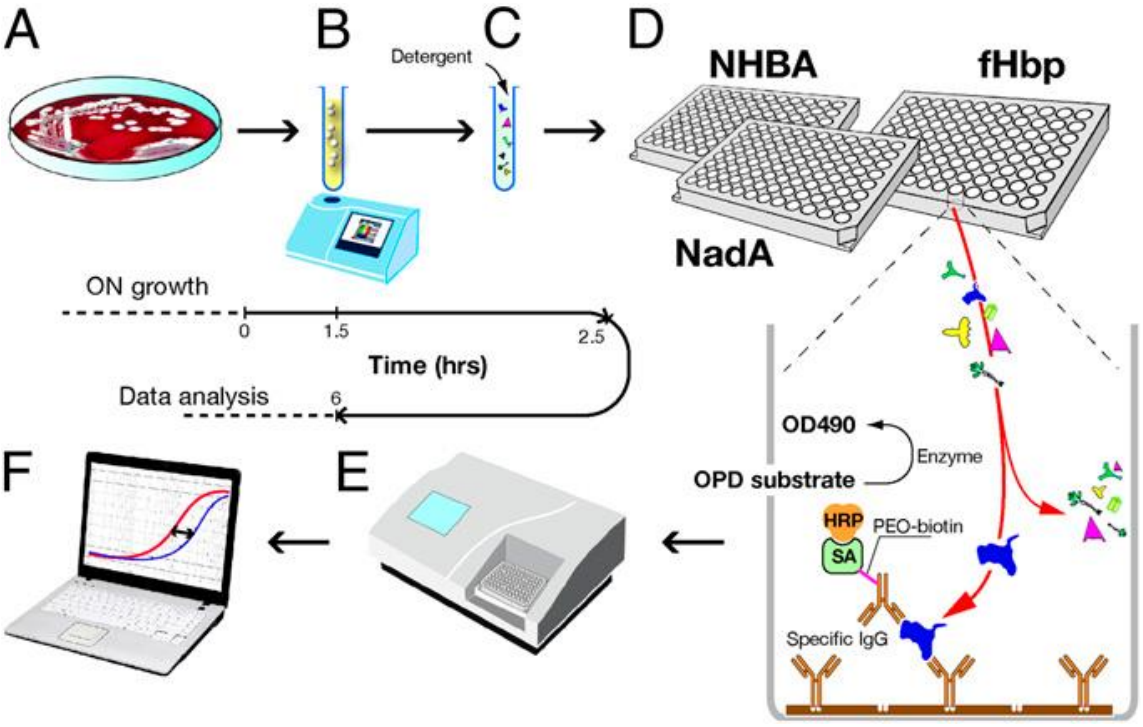

Figure 7. Schematic of the MATS ELISA method. (**A**) MenB bacteria are grown overnight on chocolate agar. (**B**) A suspension of bacteria taken from the plate is prepared to a specified OD_{600}. (**C**) Detergent is added to the suspension to extract the capsule and expose the antigens. (**D**) Serial dilutions of extract are tested in the MATS ELISA. A specific capture antibody (yellow) binds one of the antigens (example: fHbp, blue) from the extract, which is then detected with a specific biotin-labeled antibody (yellow and purple) and a streptavidin–enzyme conjugate (green and gold). (**E**) Plates are read at 490 nm in an ELISA reader. (**F**) Results are calculated by comparing the curve of OD_{490} vs. dilution obtained with the serially diluted unknown strain to a serially diluted reference strain tested in the same ELISA plate (from [136]).

2.7 Aims of the study

The aim of this work is to understand the reasons for the diverse fHbp expression levels between circulating meningococcus strains and their implications for vaccine coverage. Therefore, this work is based on the investigation of four different aspects:

- Use of selected reaction monitoring mass spectrometry to quantify fHbp levels in a panel of representative strains in an antibody-independent manner.
- Identify the sequence determinants which may drive the diverse levels of fHbp expression in circulating strains.
- Investigate the differential regulation of expression of circulating strains with different control sequence determinants.
- Generate a predictive model of fHbp expression based on sequencing of the locus which may be important for evaluation of fHbp-based vaccine coverage.

3. Results

3.1. fHbp levels correlate with bactericidal killing and serum resistance

Factor H binding protein (fHbp) is an important virulence factor of *Neisseria meningitidis* and a protective antigen able to elicit a robust immune response in preclinical and clinical testing. As such fHbp is a component of two vaccines against serogroup B meningococcus (MenB). The *fHbp* gene is present in most circulating meningococcal strains, however the level of protein expression varies considerably among isolates [85, 90].

To investigate the relationship between fHbp amounts and susceptibility to fHbp antibodies-mediated killing in immune serum, in the so-called serum bactericidal assay (SBA), we generated a recombinant strain with inducible fHbp expression. For this we used an MC58 Δ*fHbp* strain complemented with a *fHbp* gene expressing var1.1 under the control of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter. Following induction with increasing IPTG concentrations bacteria expressing incremental amounts of fHbp were obtained. The increasing amount of total protein was detected by Western blot (WB) and determined by selected reaction monitoring mass spectrometry (SRM-MS) (Figure 8A and Table 1, respectively). While with WB it was possible to detect two faint bands at the expected molecular weight of fHbp for the strains grown either without IPTG or with 0.0075 mM IPTG, the mass-spectrometry analysis did not detect any signal above the lower limit of quantification (LLOQ), which was calculated as 108 pg fHbp/µg total extract. Whereas, in the case of the wild type strain and the complemented strain induced with 0.03 and 0.1 mM IPTG the intensities of the bands corresponded to comparable fHbp amounts as measured by SRM-MS. Moreover, the differential fHbp expression on the bacterial surface was confirmed by Fluorescence Activate Cell Sorting (FACS) analysis (Figure 8B). The fluorescent intensities measured increased comparably to the increased level of expression measured in the WB, however the maximum surface fluorescence was measured in the wildtype strain albeit that the complemented strain induced with 0.1 mM IPTG exhibited higher bands of fHbp expression by WB. The reason for this was not investigated further.

We then measured the ability of anti-fHbp antiserum from mice to mediate complement-dependent killing of the isogenic bacteria expressing different levels of fHbp on the surface

through the SBA. This assay, which measures the dilution of the immune serum which kills 50% of the bacteria or the bactericidal titer, was performed in the presence of antibodies against the homologous var1.1 and rabbit complement. Bacteria expressing levels below LLOQ were not killed (titers < 16), suggesting that the amount of protein was not sufficient to mediate killing of the recombinant strains by homologous serum (Table 1). Bacteria induced with 0.03 and 0.1 mM IPTG exhibited high bactericidal titers of the anti-fHbp antiserum, 4096 and 16384, respectively. These results suggest that there is a certain minimal threshold of expression of fHbp on the surface necessary for efficient killing by fHbp-immune serum, and that above this threshold higher levels induce more effective killing.

After showing that specific fHbp Ab-mediated killing of the strains in the presence of serum as source of complement was correlated with fHbp amounts exposed on the surface of the bacteria, we investigated the influence of the protein to mediate generic evasion from complement-mediated killing. As stated in the introduction, it has been demonstrated that fHbp binds human Factor H (hfH), a key down-regulator of the complement alternative pathway, thus enabling the bacterium to evade complement-mediated killing and to survive in human blood [35, 141]. As a consequence, meningococci with less hfH bound to their surface are more susceptible to complement-mediated bacteriolysis [142]. The same set of strains used with the SBA assay was incubated for three hours with 40% human serum in the presence of the corresponding IPTG concentrations and samples were plated every 60 min for the CFU count. Figure 8C reports the results of a single serum resistance experiment; however, three replicates were performed and the trend of the curves was similar (data not shown). As shown, the survival of the strains after three hours of incubation reflected the profile of expression on the surface of the bacteria observed by FACS. Therefore, fHbp amounts were correlated with the ability of the bacterium to survive within human serum from non-immune individuals (individuals which had not received a vaccine containing fHbp antigen), while the amounts of fHbp was inversely correlated to the ability of the strain to survive fHbp antibody- mediated killing by mice immune serum.

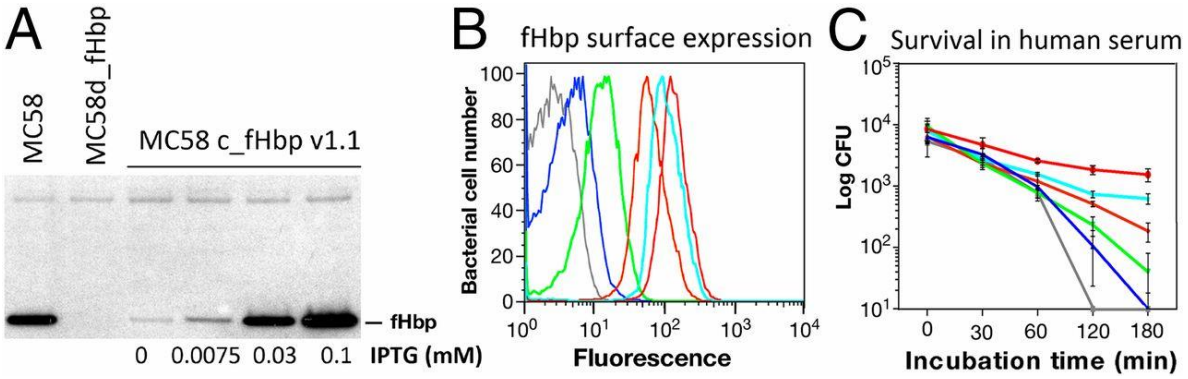

Figure 8. Quantification of fHbp in recombinant MC58 strains. (**A**) Western blot analysis with anti-fHbp var1.1 polyclonal mouse antisera on MC58 WT wild type, MC58 Δ*fHbp*, and MC58 Δ*fHbp* strain complemented with *fHbp* var1.1 induced with increasing concentrations of IPTG (0, 0.0075, 0.03, and 0.1 mM IPTG). (**B**) FACS analysis performed using polyclonal anti-fHbp var1.1 mouse antisera against the same strains in the same conditions analyzed by Western blot. (**C**) The level of fHbp expression influences meningococcal survival in human serum. 10^4 bacterial CFU of the MC58 strains cited above were incubated with 40% human serum, in the presence of increasing concentrations of IPTG. Bacterial survival was monitored for 180 min. Red: MC58 wild type; gray: MC58 Δ*fHbp*; blue: MC58 c_fHbp v1.1 (IPTG 0 mM); green: MC58 c_fHbp v1.1 (IPTG 0.0075 mM); orange: MC58 c_fHbp v1.1 (IPTG 0.03 mM); cyan: MC58 c_fHbp v1.1 (IPTG 0.1 mM).

			SBA results	
Strain	IPTG concentration, mM	fHbp amount, pg/µg total extract	Anti-var1.1	Anti-capsule*
MC58 c_fHbp	0	<LLOQ	<16	>65536
MC58 c_fHbp	0.0075	<LLOQ	<16	>65536
MC58 c_fHbp	0.03	526.10	4096	>65536
MC58 c_fHbp	0.1	856.08	16384	>65536

Table 1. SRM-MS data and SBA titers obtained for MC58 expressing increasing amount of fHbp var1.1 as a result of induction with different IPTG concentrations. Baby rabbit serum was used as source of exogenous complement. Positive titers are considered as titers >16. *An anti-serogroup B capsular monoclonal antibody (SEAM 12) was used as control of the experiment.

3.2. Sequence variability within the *cbbA-fHbp* intergenic region may account for the differential level of fHbp expression observed in a selected panel of 105 strains

It has been well documented that natural circulating strains of meningococcus exhibit very different levels of fHbp protein production [85, 90]. Given the importance of the surface expression of fHbp for the fitness of the bacterium and its susceptibility to fHbp-antibodies, we focused our attention on the possible genetic factors leading to this variability in expression levels of fHbp in circulating strains. A panel of 105 isolates was selected composed of strains expressing each of the variants 1, 2 and 3. Within each variant, 35 strains were selected to reflect the relative frequencies of the five most prevalent fHbp subvariants (var1.1, 1.13, 1.14, 1.15 and 1.4 for variant 1; var2.16, 2.19, 2.21, 2.24 and 2.25 for variant 2; var3.29, 3.30, 3.31, 3.45 and 3.47 for variant 3), as well as the genetic diversity of serogroup B meningococcal strains.

Likely most regulatory elements involved in the control of fHbp expression identified to date are found within the intergenic region between *fHbp* and its upstream gene *cbbA*, and include a transcriptional terminator directly downstream of the *cbbA* gene, a FNR transcriptional factor binding site, a dedicated *fHbp* promoter [90], and a region downstream of the transcriptional start site that has been hypothesized as an RNA thermosensor [98] (Figure 9).

To investigate the sequence variability within the intergenic region upstream of the *fHbp* gene, this region was amplified by PCR and sequenced for the 105 strains under investigation. We analyzed a region spanning from the stop codon of *cbbA* to 27 nt downstream of the putative initiation codon mapped by Masignani and collaborators [72] to include the nucleotides of the proposed region in the thermoregulation of fHbp [98] (Figure 9).

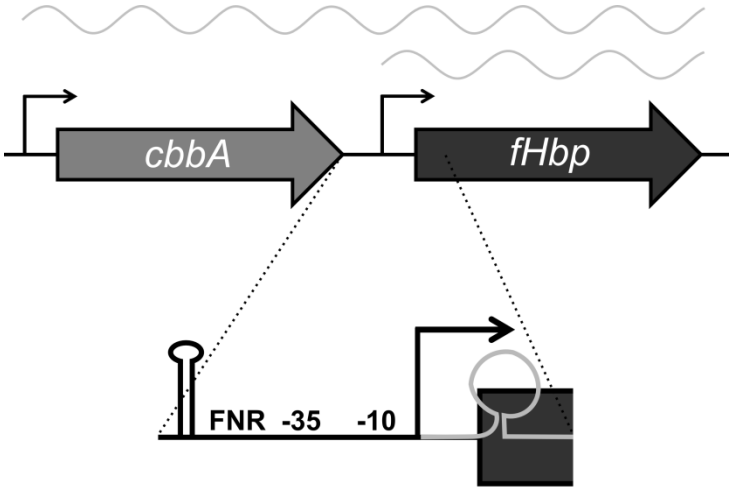

Figure 9. Schematic representation of the region under investigation and regulatory elements previously identified. The *cbbA* and *fHbp* genes are represented in grey and dark grey, respectively, with the possible transcripts of *fHbp* identified by Oriente and colleagues [90] depicted above the genes. In the lower panel the intergenic region is enlarged and the regulatory elements are listed and represented as cartoons.

Two strains belonging to subvariant 2.19, LNP24622 and M07-0240852, contained an insertion sequence (IS30) located 73 nt downstream from the stop codon of *cbbA* and were not considered in the further multiple sequence alignment analysis. A phylogenetic tree was constructed from the multiple sequence alignment of the 103 sequences by Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. The tree allowed the identification of 8 major promoter clades (Figure 10A). Mapping the fHbp coding sequence variants of each strain onto this tree indicated that variant 1 and variant 3 expressing strains have intergenic sequences that segregate largely into 4 and 3 diverse promoter clades, respectively, that interestingly do not cluster together according to the coding sequence. In fact, var3 sequences present in clades VI and VII are similar; whereas, clade VIII is genetically closer to clades IV and I, which are largely associated with var1, and conversely clade II is genetically distinct from the other var1-associated clades. Whereas, most of variant 2 sequences have intergenic regions grouped into the same promoter clade (Figure 10A). Most of the strains of var1 were associated to clades I to IV. As shown in Figure 10B, clade I contained mostly var1.1, clade II contained mostly var1.15, clade III contained mostly var1.4 and clade IV contained mostly var1.13 and 1.14. Interestingly, most of the strains harboring var2, irrespective of their subvariant, were found in clade V (Figure 10B).

Finally, clades VI, VII and VIII contained strains expressing fHbp subvariants 3.45, 3.31 and 3.47, respectively. This suggests that for var1 and var3, each subvariant can generally be associated with a single promoter clade, in line with the close linkage of the sequences; however, this is not always the case because within the same clade more variants can be found, as seen in Figure 10B.

Selected Reaction Monitoring Mass Spectrometry (SRM-MS) was employed for the targeted quantification of fHbp in the panel of 105 strains [143]. Quantification of fHbp was performed on lysates of the 105 strains grown overnight on chocolate agar plates and ninety of them provided a SRM signal above the lower limit of quantification (LLOQ), which was calculated as 108 pg fHbp/µg total extract. Overall these results showed that the level of fHbp expression was indeed considerably variable across the 105 strains. When the absolute amount of fHbp measured in each strain was plotted against the clades of the intergenic region, we found that the protein expression was associated with the promoter clade (Figure 10C). Clades I and II, containing mostly var1.1 and var1.15, showed the highest median SRM-MS quantities (411.93 and 608.73 pg/µg, respectively), while 7 out of 13 strains associated with clade VI and carrying fHbp var3.45 had fHbp values below the LLOQ (113.57 pg/µg). The other clades associated with variant 1 fHbp (clades III and IV) had medians of 258.8 and 152.21 pg/µg, respectively; whereas clades VII and VIII, both associated to var3 had median values of 148.64 and 136.92 pg/µg, respectively. Interestingly, isolates containing var2 fHbp from all the 5 subvariants included in this panel of strains were within clade V and had very similar and low amounts of the protein (156.17 pg/µg).

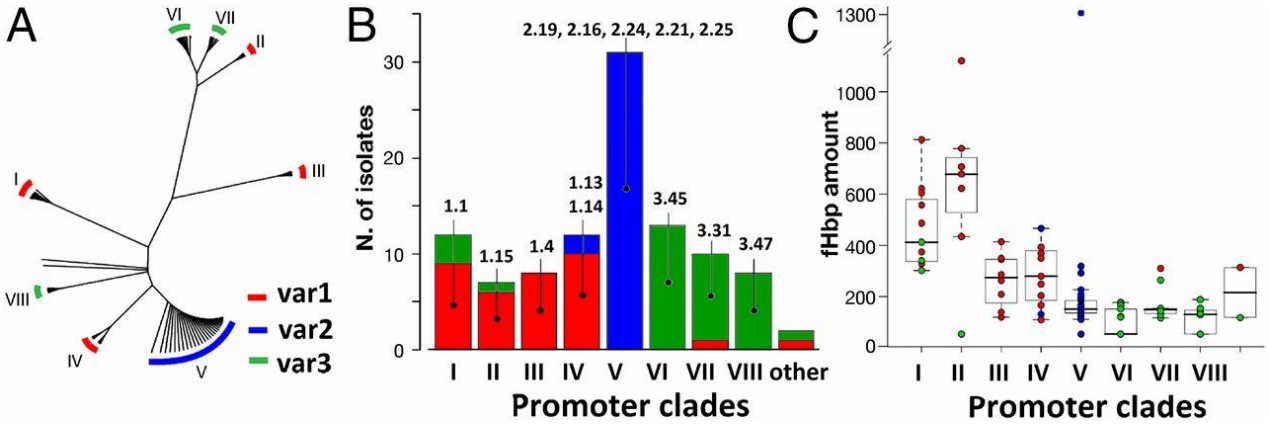

Figure 10. Association between promoter clades and fHbp variants and subvariants. (**A**) UPGMA-generated phylogenetic tree obtained from the multiple sequence alignment of the *cbbA–fHbp* intergenic region of the 103 strains under investigation. Clades I to VIII were numbered progressively to reflect the prevalence of the main subvariants included. Association between clades and var1, var2, and var3 fHbp is indicated in red, blue, and green, respectively. (**B**) Histogram showing association of clades I to VIII with specific fHbp subvariants. Subvariants associated with each clade are indicated above each bar. (**C**) Boxplots showing the distribution of SRM-MS values of different strains clustered by promoter clades. Thick bars indicate the median SRM-MS value for each group, each box delimits the interquartile range, and the whiskers mark the 95% frequency intervals of SRM-MS values.

3.3. Identification of the predominant fIR type elucidates the major genetic differences between promoter clades

Within each promoter clade we identified the most representative unique sequence (here named as *fHbp* Intergenic Region - fIR - type) (Figure 11A). Clade I contained nine out of 14 strains with an identical sequence, the fIR7. Polymorphisms in this region were found mostly in the region proposed as an RNA thermosensor (Figure 11B). Within clade II, four strains harbored the same sequence, fIR type 1, whereas the other three strains contained SNPs in the Rho-independent terminator region and in the spacer between the -35 and -10 boxes. Interestingly, all the strains within clade III had a conserved intergenic region, fIR2. In the case of clade IV two different intergenic region types were taken into account, fIR types 3 and 6, since there was no clear representative sequence (five and six strains for fIR 3 and 6, respectively). The two intergenic regions differed only for two SNPs located in the RNA thermosensor. Surprisingly, most of the strains within clade V shared the same sequence, fIR4, with the exception of three out of 31 strains that contained SNPs in the -35 box or in the RNA thermosensor sequence. Both clades VI and VII contained very diverse sequences and several SNPs were observed within the Rho-independent terminator or the RNA thermosensor sequences; the most representative sequences were fIR types 20 and 15, respectively. Conversely, all strains within clade VIII maintained the same intergenic region, fIR16. The nine fIR types identified represent 77% of the strains under investigation; since we are considering only identical sequences, the number of strains grouped into the category “Others” is 23%.

The data on quantification of fHbp by SRM-MS were replotted in relation to the fIR types for each strain (Figure 11C). We then evaluated for both boxplots (Figure 10C and Figure 11C) the interquartile range (IQR), which is the difference between the third and the first quartile of the

boxplot and an indicator of the variability of the data set (Table 2). By comparing the IQR obtained when plotting fHbp amounts with the respect of promoter clades or fIR sequence types, the implementation of the fIR classification allowed us to restrict the range of variability of fHbp expression levels, with the exception of fIR1 and 7. Interestingly, this was the case especially of strains belonging to var3 which showed the higher degree of variability within the DNA sequence of the *fHbp* intergenic region. Notably, the two intergenic regions, fIR3 and 6, that cluster within the same promoter clade and contained polymorphisms only within the RNA thermosensor region showed significantly different levels of expression. Therefore, while fewer strains might be included, this analysis would be more accurate and precise.

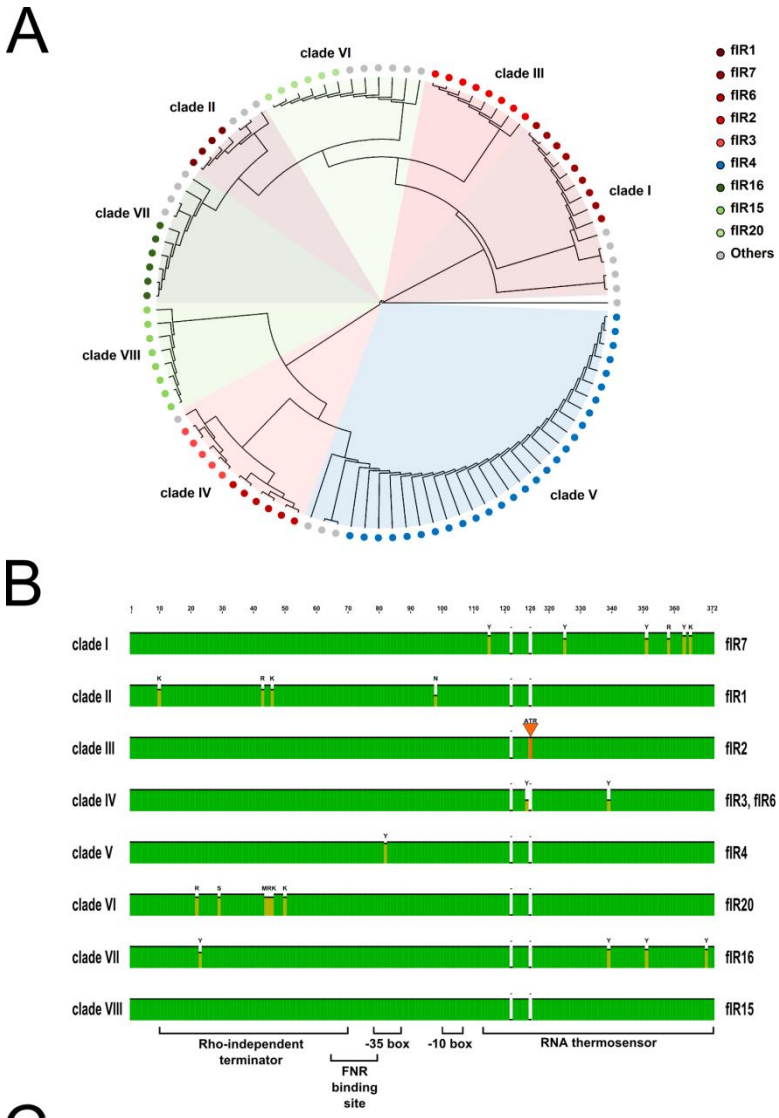
A
clade VI
clade III
clade II
clade I
clade VII
clade VIII
clade IV
clade V
fIR1
fIR7
fIR6
fIR2
fIR3
fIR4
fIR16
fIR15
fIR20
Others
B
clade I
clade II
clade III
clade IV
clade V
clade VI
clade VII
clade VIII
fIR7
fIR1
fIR2
fIR3, fIR6
fIR4
fIR20
fIR16
fIR15
Rho-independent terminator
FNR binding site
-35 box
-10 box
RNA thermosensor
C
fHbp amount
(pg fHbp/µg total extract)
2000
1500
800
500
400
300
200
100
fIR1
fIR7
fIR6
fIR2
fIR3
fIR4
fIR16
fIR15
fIR20
clade II
clade I
clade IV
clade III
clade IV
clade V
clade VII
clade VIII
clade VI
Sequence types

Figure 11. Association between fHbp intergenic region types and fHbp variants. (**A**) Phylogenetic tree obtained from the multiple sequence alignment of the *cbbA–fHbp* intergenic region of the 103 strains under investigation. Promoter clades from the previous analysis are represented as colored sectors inside the tree. Colors of the fIR types reflect the association with var1, var2, and var3 fHbp (shades of red, blue, and green, respectively). (**B**) Multiple sequence alignments of each clade. The most representative sequence (the fIR type) is indicated on the right. Green areas indicate nucleotides with 100% identity, and variable regions are indicated in yellow. Polymorphisms are indicated as Y (pyrimidine, C or T), R (purine, A or G), K (keto, G or T), M (amino, A or C), S (strong, C or G), and N (any nucleotide). Gaps are indicated with "-". Within clade V one sequence contains a nucleotide (T) at position +122 of the alignment that is not present in any of the other sequences analyzed. At position +128 of the alignment the presence of the ATR insertion element in clade III is indicated. (**C**) Boxplots showing the distribution of SRM-MS values of different strains clustered by sequence types. Thick bars indicate the median SRM-MS value for each group, each box delimits the interquartile range, and the whiskers mark the 95% frequency intervals of SRM-MS values. Expression values of the strains belonging to the clades but not to the fIR types (Others) are plotted as grey points and are not considered in the boxplot.

	Median	First quartile	Third quartile	IQR
clade I	411,93	340,22	521,16	180,94
fIR7	459,30	326,66	564,97	238,31
clade II	608,73	440,92	726,70	285,78
fIR1	704,31	547,13	940,56	393,44
clade III	258,80	159,28	324,49	165,21
fIR2	258,80	159,28	324,49	165,21
clade IV	237,13	152,21	326,85	174,63
fIR6	324,87	293,01	375,86	82,85
fIR3	166,53	128,41	197,68	69,27
clade V	156,17	135,45	198,16	62,71
fIR4	155,94	135,55	194,55	58,99
clade VI	113,57	108,00	138,58	30,58
fIR20	108,00	108,00	108,00	0,00
clade VII	148,64	120,41	243,84	123,43
fIR16	137,72	108,00	154,63	46,63
clade VIII	136,92	108,00	215,54	107,54
fIR15	136,92	108,00	215,54	107,54

Table 2. Values calculated from the boxplots in Figure 10C and in Figure 11C. Median, first quartile and third quartile of fHbp amounts plotted according to either the clades or the fIR sequence types were extracted from the boxplots showed above. IQR, interquartile range, is calculated as third - first quartiles and is an indication of the variability of the set of data. The larger the IQR, the more variable the data set is.

In an attempt to elucidate the differences at the DNA level that might be responsible for the variability in fHbp amounts produced by bacteria, we aligned the sequences of the nine intergenic regions, putting in evidence the regulatory elements therein, and looked for SNPs that could be at the basis of the differences in fHbp expression (Figure 12). In details, six fIR types maintained

the same Rho-independent terminator sequence represented by a 'perfect' palindromic 20 bp sequence followed by 5 Ts [144]; whereas the other three, fIR1, 7 and 16, contained multiple SNPs within one of the palindromic sequences. Interestingly, when we estimated the free energy predictions of each one of the Rho-independent terminator sequences of all the intergenic regions [145], fIR types 1, 7 and 16 had very low or medium free energy (ΔG = -13.0; -14.8 and -24.7, respectively) (Figure 13D, C and B, respectively); whereas, the other fIR types contained the same strong terminator with ΔG = -27.3 (Figure 13A). Indeed, through a visual analysis of the secondary structures of the RNA [146] it was possible to locate the identified SNPs within the stem of the terminator; their presence would impair complementarity of base-pairing within the palindromic stem and possibly its structure and function, and may result in less efficient termination and hence read through of the RNA polymerase from the upstream gene *cbbA*. The sequences of both the FNR binding site and the -35 box were 100% conserved through all fIR sequence types; however, the outlier strain M08-0240104, belonging to clade V and differing from fIR4 for solely a SNP in the -35 box had the highest absolute fHbp expression (1681.25 pg fHbp/µg total extract). Three different SNPs were identified within the spacer between the -35 and -10 boxes. Differences in spacing between -10 and -35 elements have been associated with different promoter strengths. Moreover, two alleles of the -10 box were found, TACCAT or TACCGC. Interestingly, two out of three sequences were associated with a weak or medium Rho-independent terminator. Furthermore, an insertion element of 187 bp rich in A and T (ATR) [72, 90, 95] was identified in the fIR2, 14 nt downstream the mapped RNA transcriptional start site [90]. The highest degree of variability was observed within the long region described as putative RNA thermosensor [98]. Two polymorphisms at positions 115 (C/T) and 142 (T/C) of the multiple sequence alignment segregates with the -10 alleles TACCGC/TACCAT, respectively. Notably, a polymorphism (T/C) was found just downstream the putative ribosome binding site (AGGAG). Only two polymorphisms segregate fIR types 3 and 6, one in position 126 (T/C) and one in 151 (T/C). In summary, by identifying the major sequence type within each of the clades we have identified the main genetic polymorphisms differentiating one clade from the next which may determine the differences in fHbp expression levels seen from strains harbouring these intergenic sequences.

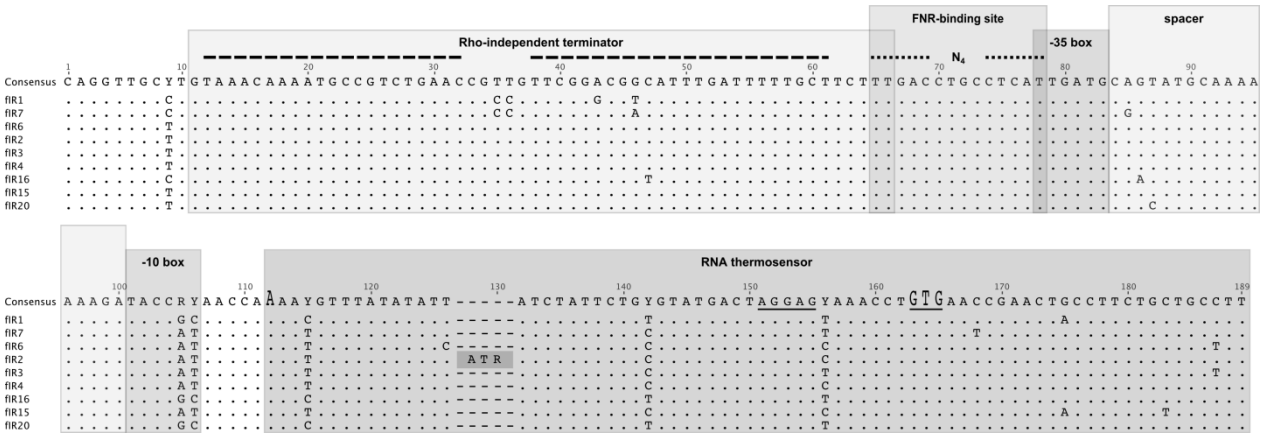

Figure 12. Multiple sequence alignment of the nine fIR types. The consensus sequence is at the top of the aligned sequences. Dots represent conserved positions and mismatches are indicated with nucleotides. The regions of the regulatory elements are indicated and boxed in shades of grey. Palindromic sequences of the stem of the terminator are dashed. The mapped FNR-binding site is indicated as dotted lines. The insertion sequence rich in A and T (ATR) is boxed. The transcriptional start site is indicated at position 112 and the RBS –AGGAG– and translational start site GTG at position 143 are underlined.

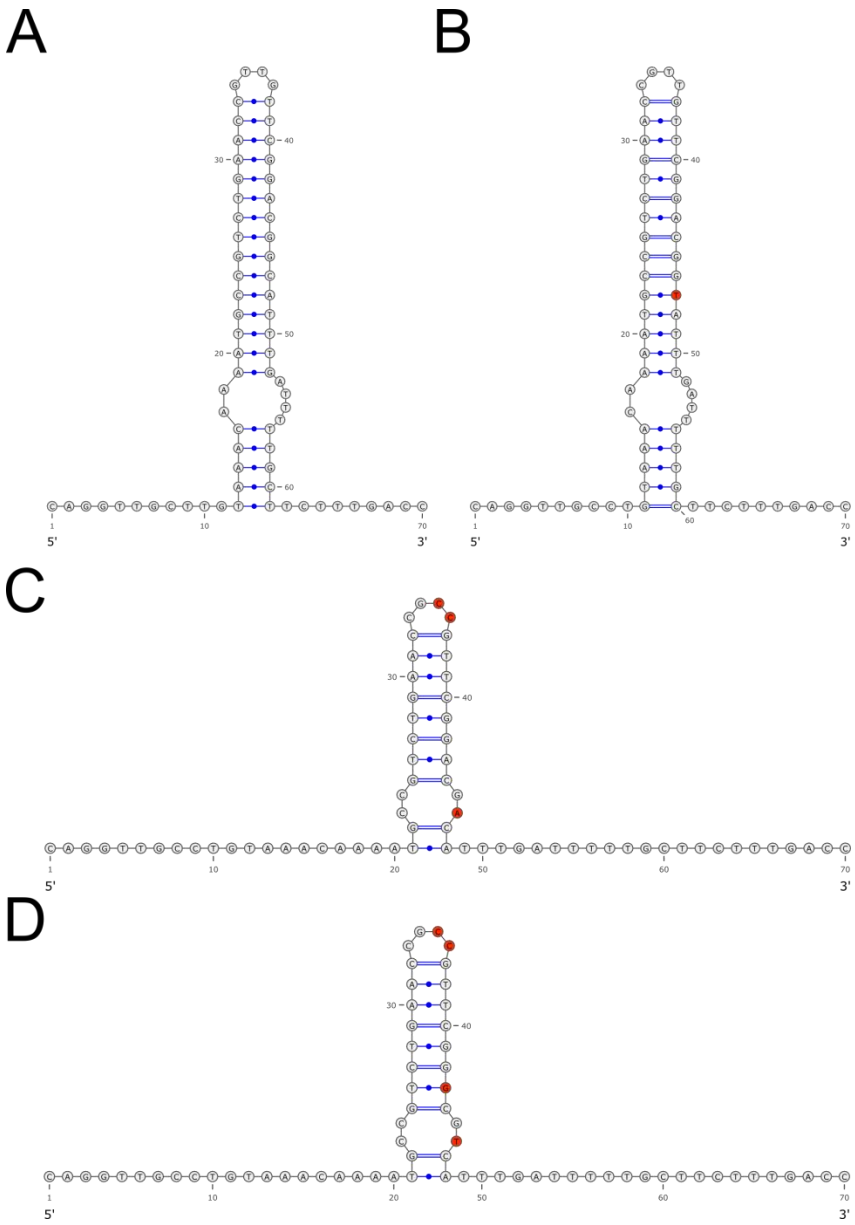

Figure 13. Schematic representation of the secondary structures of the Rho-independent terminators. The structures of the terminators with ΔG = -27.3 (**A**), -24.7 (**B**), -14.8 (**C**) and -13.0 (**D**) are represented. SNPs from the strong terminator sequence are highlighted in orange.

3.4. fIR sequence type is predictive of *in vitro* expression levels in an isogenic background

To confirm the hypothesis that the amount of fHbp produced by the different bacterial strains is determined by the DNA sequence of the intergenic region of *fHbp* we generated a panel of isogenic recombinant strains where the same fHbp variant was under the control of the nine fIR types identified (Figure 14A). The intergenic regions under investigation were amplified and cloned upstream of the coding sequence (CDS) of fHbp var1.1 into a vector carrying an antibiotic selection marker and flanked with regions for homologous recombination for replacement within the wildtype locus. The sequences of each one of the constructs were confirmed by Sanger method. Upon transformation of the MC58 Δ*fHbp* strain, an isolate for each fIR type which drives the expression of the var1.1 was selected and employed for further analyses (Figure 14A).
The panel of mutants was grown in gonococcus (GC) medium till an OD_{600} = 0.5 and total RNA was extracted. The expression levels of *fHbp* and its upstream gene *cbbA* in the set of generated mutants were tested by qRT-PCR, normalized to the reference gene *16S RNA* and compared to the strain containing the fIR7 (Figure 14B). Furthermore, the presence of a possible bicistronic transcript generated by the read through of the RNA polymerase from *cbbA* was assessed using primers to amplify the inter-transcript region from right upstream of the Rho-independent terminator and upstream of the dedicated *fHbp* promoter transcriptional start. As shown by Figure 14B, the level of transcripts in fIR1 resulted significantly higher whereas in fIR4, fIR15 and fIR20 significantly lower; however, in general the mRNA levels of *cbbA* were relatively comparable in most of other strains. Only in the case of fIR types 1, 7 and 16 was it possible to detect any signal from the inter-transcript region (Figure 14B, medium panel). These results indicate that read through from *cbbA* is possible only in strains belonging to fIR types 1, 7 and 16, suggesting the efficiency of the Rho-independent terminator is low in these strains, in line with the reduced complementarity within the stem and reduced free energy calculated for the Rho-independent terminator structure. Conversely, mRNA levels of *fHbp* transcript were more variable and the fIR1 and fIR7 strains in which the read through was observed showed the highest levels of fHbp transcript, together with fIR6 mutant. fIR3, fIR16 and fIR15 showed intermediate levels of transcript which were statistically lower than fIR7. The lowest expression

levels of *fHbp* were detected where transcription was under the control of the fIR types 2, 4 and especially 20. Taken together, these results suggest that the different fIR types result in distinct differences in transcript levels of fHbp. Given the fact that the genetic background of the recombinant strains is equal, the variability of expression is dependent only on the different sequences of the region upstream of *fHbp*. Three fIR types give rise to a read through from the upstream *cbbA* bicistronic transcript as well as a likely *fHbp* monocistronic from the *fHbp* dedicated promoter. A trend for higher levels of mRNA was seen for *cbbA* and for the inter-transcript in fIR1 strain suggesting that the bicistronic transcript is more stable than a monocistronic *cbbA* transcript. The lowest mRNA level was measured for fIR20 which has the TACCGC allele for the -10 box, and unlike the other two fIR types (fIR1 and fIR16) this intergenic sequence does not result in read-through from the *cbbA* upstream transcript.

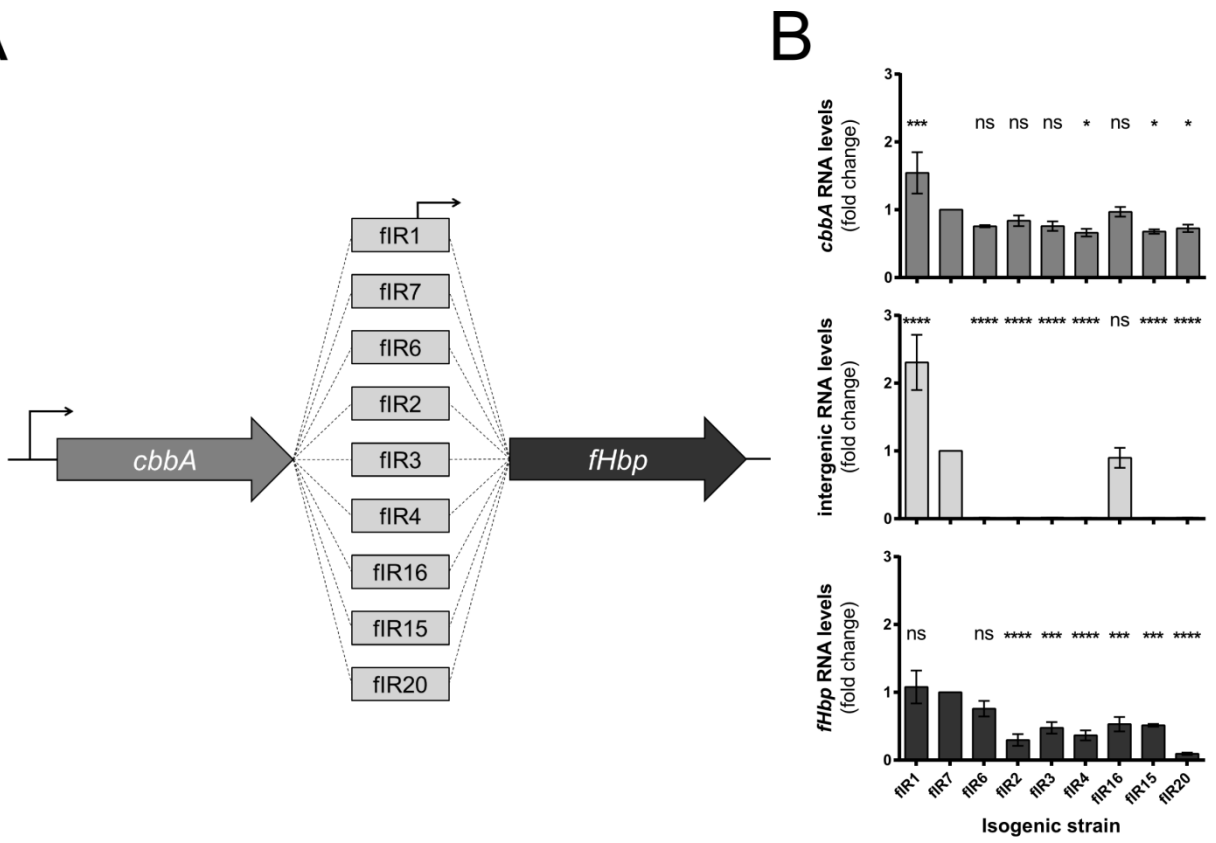

Figure 14. fIR sequence type is predictive of in vitro expression levels in an isogenic background. (**A**) Schematic representation of the isogenic recombinant mutants generated. The *cbbA* and *fHbp* genes are represented in grey and dark grey, respectively; whereas, the intergenic regions are in light grey. (**B**) Results of the qRT-PCRs

done on *cbbA* (upper panel), the intergenic region (medium panel) and *fHbp* (lower panel). Data are indicated as medians and standard deviations of three biological replicates. Values of the single replicates are normalized to the reference gene *16S RNA* and to the strain fIR7. Statistical significance is calculated with the two-way ANOVA.

We tested by Western blot (WB) the protein expression levels in the set of mutants generated, grown overnight in GC agar plates (Figure 15). The intensities of the bands at the expected molecular weight reflected the trend of expression as measured by qRT-PCR. By considering the expression levels it was possible to identify four different groups, with mutants containing fIR types 1 and 7 expressing the most (group 1) and strains bearing fIR4 and 20 the less (group 4); whereas, fIR6 and 16 strains (group 2) produced less protein than group 1, but more than group 3 (fIR types 2, 3 and 15). Notably, the two polymorphisms that segregate fIR types 3 and 6, one in position 126 (T/C) and one in 151 (T/C), appear to result in distinct level of expression although the mechanism remains unclear. The intensities of the bands in strains where fHbp was under the control of var2 and var3-associated fIR type (fIR4 for var2, fIR16, 15 and 20 for var3) were higher than what we would expect from the proteomic analysis on natural strains. Altogether these results indicate that the sequence of the *fHbp* intergenic region is associated with a range of protein produced by the bacteria but other factors are involved in the expression.

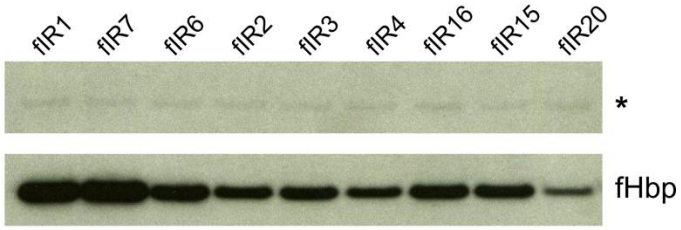

Figure 15. fHbp protein levels of the isogenic recombinant mutants. Western blot analysis on the set of mutants generated. fHbp is detected with a polyclonal serum raised against var1.1. The serum used to detect fHbp in the WB analysis also recognized unrelated protein with higher molecular weight and this cross-reactivity was used as an internal loading control (*).

3.5. Influence of the variant sequence on the expression levels of fHbp

The observed discrepancies might be caused by the different protein stability of the three fHbp variants [147, 148]. To investigate this hypothesis we generated a set of recombinant strain where

different subvariants of fHbp under the control of the same IPTG-inducible promoter were incorporated in a different locus within the MC58 Δ*fHbp* background (Figure 16A). An analysis on the steady-state levels of the *fHbp* RNA was performed by qRT-PCR on samples extracted from strains grown in GC medium with 1mM IPTG (Figure 16B). Despite a high variability between replicates, the transcript expression levels of the different strains were found to be stable, with the exception of the mutant expressing var1.1 which was significantly higher, indicating similar quantities of RNA generated. Furthermore, we quantified by SRM-MS the fHbp amounts produced by these strains grown overnight on chocolate agar plates with 1mM IPTG. Strains expressing var1.1 and var1.14 contained more fHbp than strains carrying var2 and var3 (Figure 16C). Therefore, from the same levels of transcripts, different amounts of protein were generated. Taken together these data suggest a role of the *fHbp* coding sequence either in the stability of the protein or in its translation efficiency.

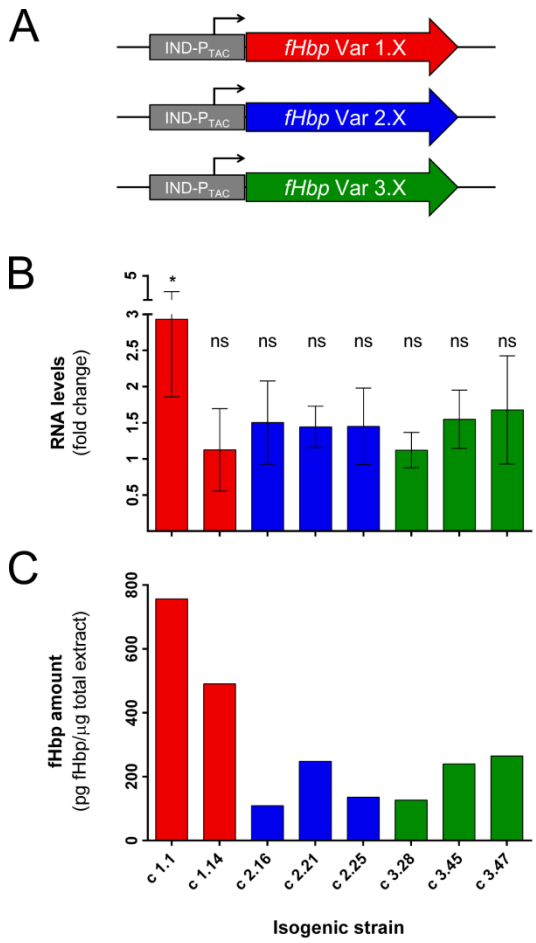

Figure 16. Influence of the variant sequence on the expression levels of fHbp. (**A**) Schematic representation of the isogenic recombinant mutants generated. The IPTG-inducible promoter is colored in grey. fHbp subvariants are colored in red, blue or green according to their variant group 1, 2 or 3, respectively. Results on the expression levels of fHbp as measured by qRT-PCR (**B**) and SRM-MS analyses (**C**) are reported. Values on the RNA levels are normalized to the reference gene *16S RNA* and to the MC58 wt strain grown till OD_{600} = 0.5 in GC with 1mM IPTG (data not shown). Statistical significance is calculated with the two-way ANOVA.

3.6. SNPs that regulate fHbp expression

To decipher the influence on the fHbp expression levels of the major genetic determinants identified between the fIR types (Figure 12) we mutagenized the intergenic region of *fHbp* in the MC58 background by site-directed mutagenesis and generated a series of isogenic recombinant strains (Figure 17A) differentiating uniquely by specific polymorphisms. We modified the sequence of the wildtype fIR7 intergenic region and the substitution of a single nucleotide within

the stem region of the terminator was sufficient to restore a correct base pairing in the stem, increasing its strength from ΔG = -14.8 to -27.3, for testing of the 'weak' and 'strong' terminator hypothesis. Both alleles of the -10 box (TACCAT or TACCGC) were generated in either the weak or the strong terminator background. In addition, all the four different variants of the spacer region were generated in the promoter with the strong terminator. Finally, in order to investigate whether the -35 SNP identified in the var2/clade V outlier strain M08-0240104 (that produced the highest amount of fHbp = 1681.25 pg fHbp/µg total extract observed in the proteomic analysis), we generated an isogenic strain with this polymorphism in the strong terminator background. The expression levels of the protein in the set of mutants generated were then tested by Western blot analysis (Figure 17B). No protein was detected at the expected molecular weight in the fHbp knockout strain (Δ*fHbp*). The *in locus*-complementation of *fHbp* with the fIR7 wild type (including the weak terminator and TACCAT -10 determinants) upstream region sequence and the downstream chloramphenicol resistance cassette restored the protein expression to the wild type levels (c*fHbp* versus wt). Notably, no differences in the band intensities were observed between the two alleles of the -10 box in the weak terminator-background (c*fHbp* [TACCAT] versus c*fHbp* -10 box [TACCGC]). When the wild type terminator (weak) was substituted with the mutated terminator with higher free energy predictions within the stem loop structure (strong) the amount of protein produced substantially decreased (c*fHbp* versus c*fHbp* term). Furthermore, this background allowed the detection of differences in the expression levels of the two -10 box-alleles; the TACCGC mutant (c*fHbp* term -10 box) which produced less fHbp compared to the TACCAT (c*fHbp* term) derivative. By comparing the different alleles of the spacer region no differences were observed (c*fHbp* term versus c*fHbp* term spacer1, 2 and 3). Interestingly, a SNP within the -35 box identified in only one strain (M08-0240104) was responsible for an extensive increase on the expression of the protein (c*fHbp* term -35 box versus c*fHbp* term).

Altogether these data indicate that identified polymorphisms in the terminator, the -10 and the -35 elements can have a significant effect on the levels of fHbp from recombinant promoters. These polymorphisms represent some of the major determinants differentiating fIR types which are naturally occurring sequences found in clinical isolates and we confirm that the presence of these SNPs within the regulatory elements of the *fHbp* intergenic region can be responsible for the variation in fHbp expression levels.

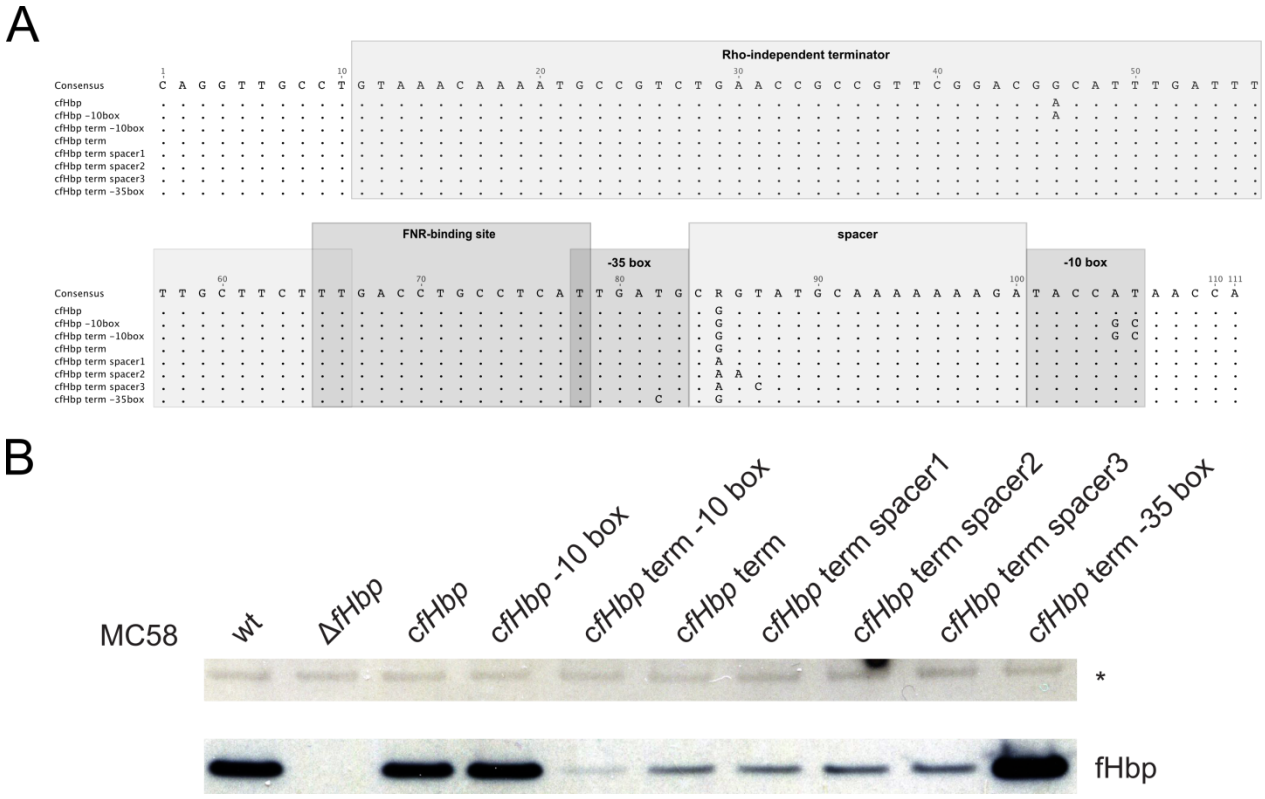

Figure 17. SNPs that regulate fHbp expression. (**A**) Multiple sequence alignment of the MC58 *fHbp* intergenic region (spanning from the translational stop codon of *cbbA* and the transcriptional start site of *fHbp*) and of the mutants of the regulatory elements generated by site-directed mutagenesis. The consensus sequence is at the top of the aligned sequences. Dots represent conserved positions and mismatches are indicated with nucleotides. The regions of the regulatory elements are indicated and boxed in shades of grey. (**B**) Western blot analysis on the set of mutants generated. fHbp is detected with a polyclonal serum raised against var1.1. The loading control is indicated with an asterisk.

3.7. Transcriptional responses to environmental conditions of the different fIR types

From previous studies, oxygen and iron availability have been implicated in regulation of fHbp expression levels in certain strains [90, 95]. In order to investigate the effect of external stimuli encountered by *Neisseria meningitidis* on *fHbp* transcription from different fIR sequence types we compared expression levels on the isogenic recombinant strain panel in the presence or absence of either oxygen or iron. The strains were grown till exponential phase in GC medium and then exposed to aerobic conditions or oxygen limitation conditions for 30 min, respectively.

The presence of a bicistronic transcript was also assessed for both oxygen and iron stresses (data not shown) and results confirmed what was already observed, that only fIR types 1, 7 and 16 resulted in inter-transcript amplification under all conditions. Expression levels of *fHbp* and *cbbA* were detected by qRT-PCR and normalized to the reference strain fIR7 (Figure 18).

The relative differences between transcript levels for each sample under aerobic vs oxygen limitation are shown in Figure 18 (B and D *fHbp* and *cbbA*, respectively) to highlight the relative responses to these conditions in each strain. Standard deviations indicate a high variability among replicates. Notably, it was higher in the presence of bicistronic transcript. Due to this variability no significant difference was observed between the two conditions, for both genes; however, a trend of increase of *fHbp* transcripts in the absence of oxygen was observed, with the exception of fIR1, (Figure 18A and C).

The effect of iron availability was tested by treating exponential phase cultures with or without 250 µM 2'2'-dypiridyl for 30 min (Dypiridyl). Data on the expression levels of *fHbp* and *cbbA* were treated similarly to the oxygen condition (Figure 18E, F, G and H). Interestingly, iron depletion did not cause any major changes in the transcript levels of *cbbA* (Figure 18G and H), and excluding fIR1, which exhibited an increase upon iron limitation, *fHbp* expression decreased in all strains (Figure 18D and E). However, in fIR2, 16 and 20 mutants the decrease was not statistically significant. Altogether these results indicate a different regulation of the promoters of both *cbbA* and *fHbp*, which in turn might influence differently the antigen expression levels, depending on the presence of the bicistronic transcript. Notably, *cbbA* higher stability in the fIR1 mutant dampens the *fHbp* dedicated promoter regulation.

A role of temperature in the regulation of fHbp has been proposed [98]. Indeed, the fIR types identified exhibit different RNA secondary structures (data not shown) that might be involved in thermoregulation of this antigen. However, in our experimental conditions we never observed differences in the protein amounts between 30 and 37°C (data not shown).

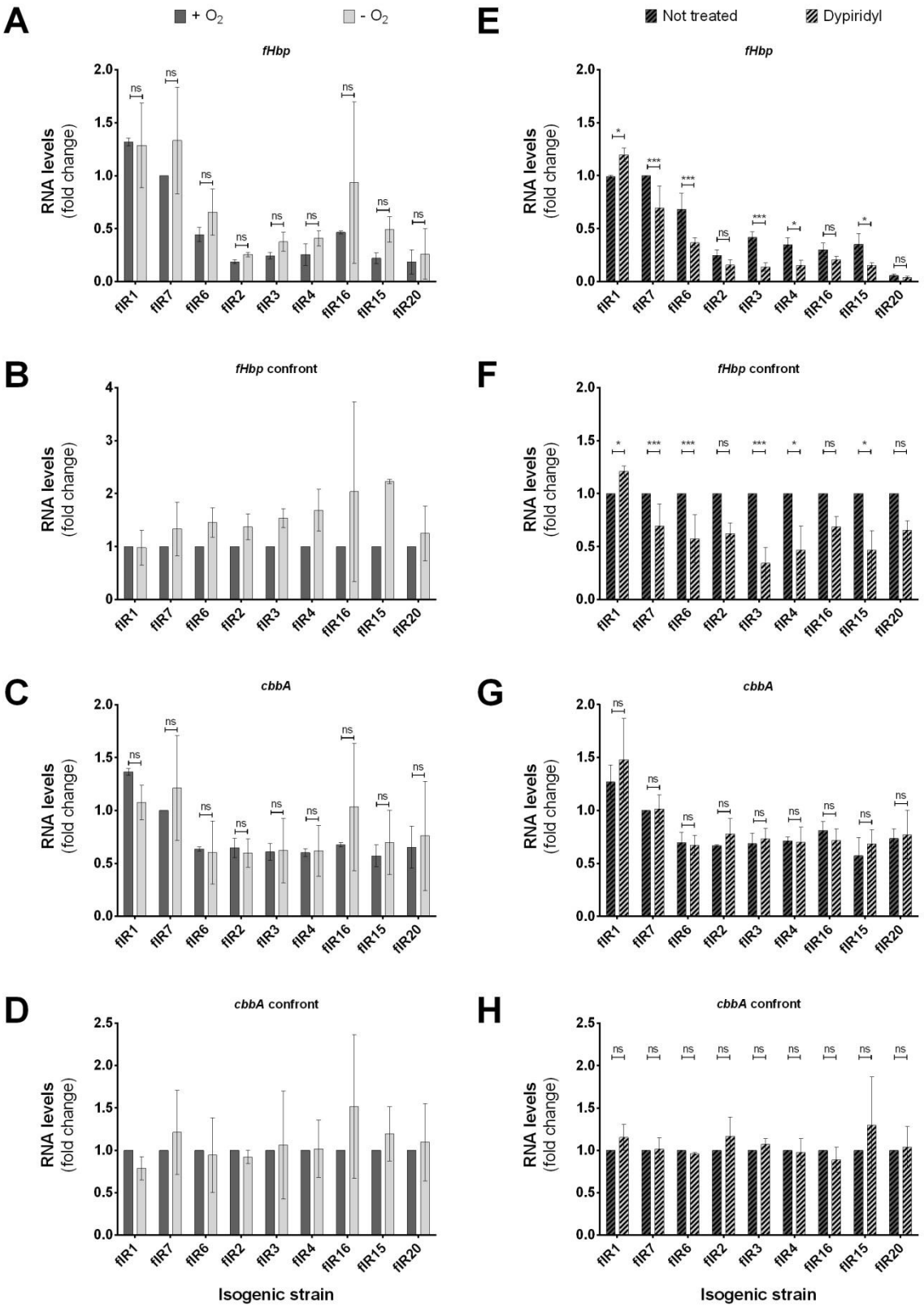
A
+ O2
- O2
fHbp
RNA levels (fold change)
fIR1
fIR7
fIR6
fIR2
fIR3
fIR4
fIR16
fIR15
fIR20
B
fHbp confront
C
cbbA
D
cbbA confront
Isogenic strain
E
Not treated
Dypiridyl
F
G
H

Figure 18. Response to stress of the different fIR types measured by qRT-PCR. Values represent medians and standard deviations of three biological replicates. Data of the single replicates are normalized to the reference gene *16S RNA*. During oxygen limitation differences in the expression levels of *fHbp* (**A**) and *cbbA* (**C**) were quantified and they are reported normalized to the + oxygen condition of the fIR7 strain or of each strain. (**B** and **D**, respectively) Bars are dark grey for + oxygen and light grey for – oxygen. Results on *fHbp* (**E**) and *cbbA* (**G**) expression levels during iron starvation are normalized to the not treated (ethanol was added to the culture as dypiridyl is dissolved in it) condition of the fIR7 strain or to the not treated condition of each strain (**F** and **H**, respectively). Dashed bars are dark grey for not treated (+ iron) and light grey for dypiridyl (iron depletion). Statistical significance is calculated with the two-way ANOVA.

3.8. Influence of the fIR types on the bactericidal killing and serum resistance

Having observed the correlation between fHbp quantity and both anti-fHbp mediated bactericidal killing and the ability of the bacteria to survive within serum, we investigated the biological significance of different amounts of protein expressed by the natural fIR types identified. The strains were grown in Müller-Hinton broth plus 0.25% glucose till OD_{600} = 0.25 and tested for both SBA and human serum survival. Initially, to confirm that fHbp expression levels was maintained under different growth conditions a Western blot analysis was performed on total cell extracts collected at the end of the growth. As Figure 19A shows, the same grouping determined by the expression levels of the isogenic recombinant strains was obtained under these growth conditions (fIR1/7 > fIR6/16 > fIR2/3/15 > fIR4/20, groups 1, 2, 3 and 4, respectively) with the respect of what already observed from strains grown overnight in GC agar plates (Figure 15).

To assess the capability of antibodies raised against fHbp var1.1 to mediate killing of these strains we performed a serum bactericidal assay (Figure 19B). In these experiments human plasma was employed as source of complement to be more physiological. The titers obtained from this analysis were generally lower with the respect to that observed previously with Baby Rabbit Complement, which is in line with the presence of hfH (Table 1). Interestingly, by comparing the SBA titers obtained it was possible to group the strains into the same categories identified by WB. Indeed, mutants expressing high amounts of the protein (group 1) were killed by lower dilution of serum against fHbp and vice versa, in the case of group 4. Hence, in our experimental conditions bactericidal killing correlated with fHbp expression levels determined by fIR type natural promoters. We then tested the same set of mutants for the capacity of surviving

in human serum. After the growth the strains were incubated in 60% serum for three hours. The percentages of survival of each mutant at the end of the experiment are reported in Figure 19C. As negative control a MC58 strain lacking the *fHbp* gene (Δ*fHbp*) was employed and the results indicate that the strain did not survive after incubation with human serum, as already reported [70, 78]. Differently to what observed in the SBA, a diverse grouping was identified. Indeed, the fIR1 strain, which expresses a high amount of fHbp, survived better than all the other mutants; whereas, fIR3, 4 and 15 were almost killed in three hours. Moreover, no statistically significant difference was observed between fIR7, a high expressor similar to fIR1, and fIR2, 16 and 20 strains. Interestingly, the intergenic region type 20 was associated with very low amount of fHbp *in vitro*. Therefore, fHbp expression levels determined by natural promoters and measured from an *in vitro* growth culture were less correlated with complement-mediated killing. These results suggest a possible regulation of *fHbp* in the presence of human serum for at least some of the fIR types under investigation.

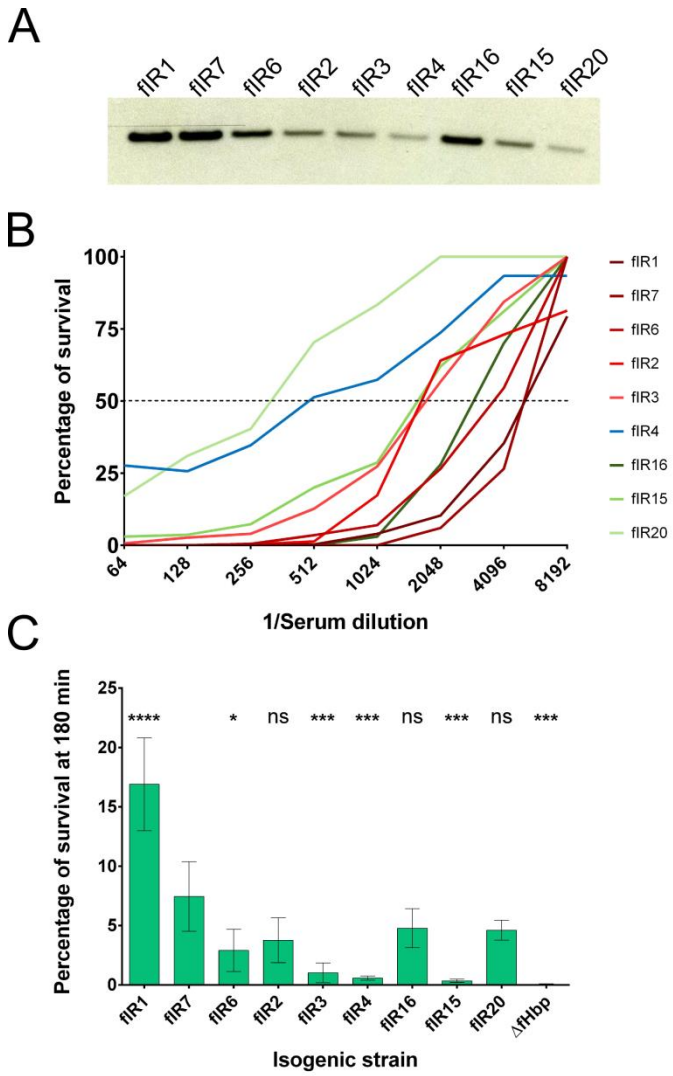

Figure 19. Influence of the fIR types on the bactericidal killing and serum resistance. (**A**) Western blot analysis on the set of the fIR types mutants grown in MH + 0.25% glucose. fHbp is detected with a polyclonal serum raised against var1.1. (**B**) Results of the SBA analysis done incubating bacteria in human plasma as source of exogenous complement and serial dilutions of antibodies against fHbp var1.1. For each strain percentages of survival are plotted according to the inverse of the serum dilution. The dashed line indicates the half of the survival from which SBA titers are obtained. fIR types are colored as shades of red, blue, and green depending on their association with var1, var2 and var3, respectively). (**C**) Percentages of survival after 3 hours incubation in 60% human serum were calculated with the respect of t_0. Data represent mean and standard deviation of three biological replicates. Statistical significance is calculated with the two-way ANOVA.

3.9. Epidemiologic analysis of the intergenic regions of *fHbp*

To extend the analysis to a more significant and wider panel of strains we extracted and aligned the nucleotide sequence of the *fHbp* gene and its upstream region from 915 isolates available in the public Bacterial Isolate Genome Sequence Database (BIGSdb, http://pubmlst.org/software/database/bigsdb/) [149]. These strains largely represent isolates of meningococcus collected in the UK between 2010 and 2012. The panel contains strains belonging to different serogroups with a majority of serogroup B (
Figure 20A), and to several CCs, including those hyper-invasive (
Figure 20B).

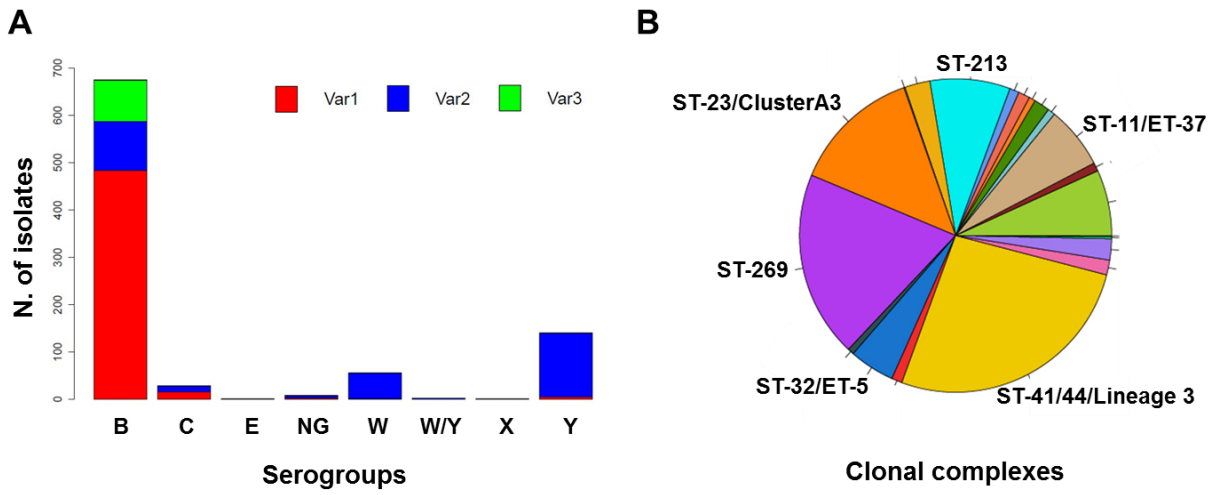

Figure 20. Panel of UK strains. (**A**) Plot of the serogroups represented in the 915 isolates. fHbp variants 1, 2 and 3 are colored in red, blue and green, respectively. (**B**) Pie chart of the different clonal complexes represented in the panel of strains under investigation. Hyper-invasive CCs are highlighted.

Given the high number of data we implemented a bioinformatics analysis to screen and categorize the intergenic regions. Each unique sequence was assigned a specific fIR type. A total of 79 different *fHbp* intergenic region types were identified; however, 11 of them represented 88.85% of the strains. Figure 21A shows the phylogenetic tree constructed from the multiple sequence alignment of the 915 sequences by Neighbor Joining (NJ) method. The nine fIR types identified in the 105 strains were among the 11 most frequently represented and two new fIR types were identified, fIR11 and 13. These are found largely in variant 1 expressing strains

(Figure 21B) and they both contain a strong Rho-independent terminator and a TACCAT -10 element. Interestingly, fIR11 is similar to fIR7, with the exception of polymorphisms within the terminator region and in positions +90, +113 and +120 of the multiple sequence alignment; whereas, fIR13 differs from fIR6 only for a SNP at position +126 of the multiple sequence alignment (Table 3).

fIR type	Rho-independent terminator	+85	+86	+87	+90	-10 box	+113	+115	+120	+126	ATR	+137	RBS	+163	+170	+178	+182
fIR1	weak	A	G	T	G	TACCGC	A	C	A	T		T	AGGAGT	C	A	C	C
fIR7	weak	G	G	T	G	TACCAT	A	T	A	T		C	AGGAGT	T	G	C	C
fIR6	strong	A	G	T	G	TACCAT	A	T	A	C		C	AGGAGC	C	G	C	T
fIR2	strong	A	G	T	G	TACCAT	A	T	A	T	ATR	C	AGGAGC	C	G	C	C
fIR3	strong	A	G	T	G	TACCAT	A	T	A	T		C	AGGAGT	C	G	C	T
fIR11	strong	G	G	T	A	TACCAT	G	T	T	T		C	AGGAGT	T	G	C	C
fIR13	strong	A	G	T	G	TACCAT	A	T	A	T		C	AGGAGT	C	G	C	T
fIR4	strong	A	G	T	G	TACCAT	A	T	A	T		C	AGGAGC	C	G	C	C
fIR16	medium	A	A	T	G	TACCGC	A	C	A	T		T	AGGAGT	C	G	C	C
fIR15	strong	A	G	T	G	TACCAT	A	T	A	T		C	AGGAGC	C	A	T	C
fIR20	strong	A	G	C	G	TACCGC	A	C	A	T		T	AGGAGT	C	G	C	C

Table 3. Polymorphisms that distinguish the 11 fIR types identified. For each of the fIR type the characteristics are listed. Definitions of "weak", "medium" or "strong" depend on the free energy prediction of the Rho-independent terminator. The numbers are referred to the position within the multiple sequence alignment. The sequence of the ribosome binding site (RBS) is underlined.

The fIR isogenic mutants in MC58 expressing var1.1 under the control of fIR11 and fIR13 were generated and the respective expression levels were tested by Western blot along with the nine original mutants (Figure 21C). By comparing band intensities, fIR11 and fIR13 promoters drive an expression of fHbp similar to fIR2 and fIR3, respectively.

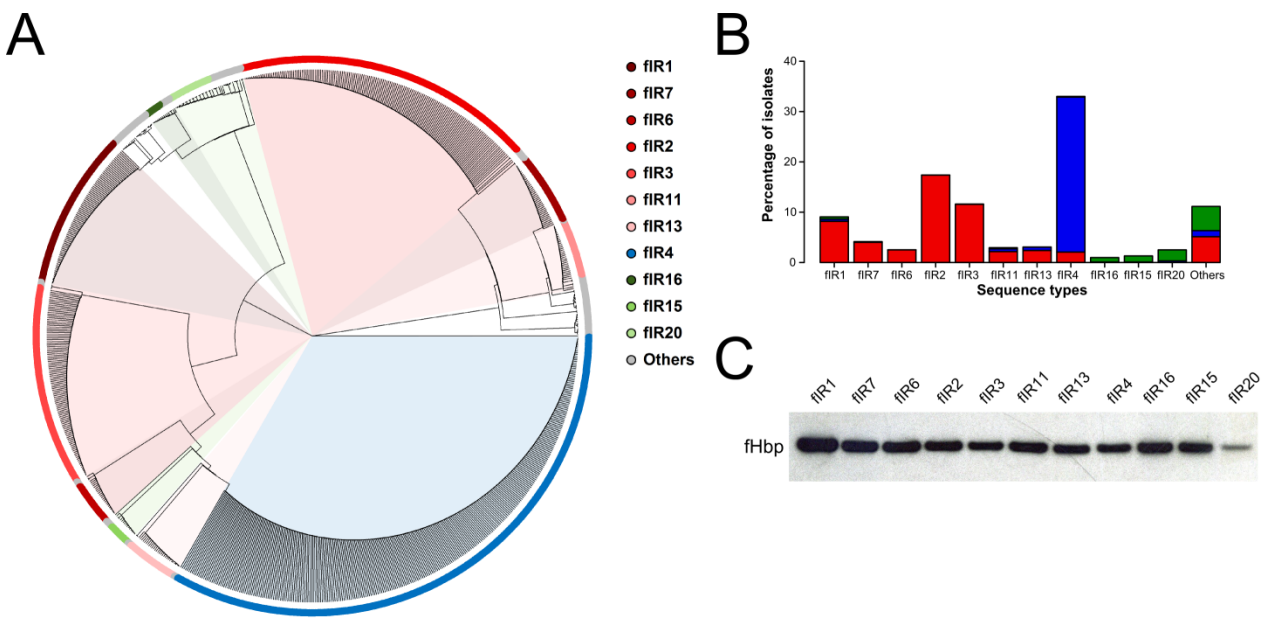

Figure 21. Association between fHbp intergenic region types and fHbp variants in a panel of 915 strains. (**A**) Phylogenetic tree obtained from the multiple sequence alignment of the *cbbA–fHbp* intergenic region of the 915 strains downloaded from the BIGSdb. Promoter clades are represented as colored sectors inside the tree. Colors of the fIR types reflect the association with var1, var2, and var3 fHbp (shades of red, blue, and green, respectively). (**B**) Histogram showing association of fIR types with fHbp variants. Strains carrying fHbp variant 1, 2 or 3 are in red, blue or green, respectively. (**C**) Western blot analysis on the fIR types mutants. fHbp is detected with a polyclonal serum raised against var1.1. The loading control is indicated with an asterisk.

fIR type	fHbp variant	fHbp subvariant	Clonal complex
fIR1	1	1.15	ST-269
fIR7	1	1.1	ST-32
fIR6	1	1.14	ST-41/44
fIR2	1	1.4	ST-41/44
fIR3	1	1.13	ST-1157, ST-60, ST-269
fIR11	1	variable	ST-11/ET-37
fIR13	1	1.14	ST-167, ST-41/44, ST-865
fIR4	2	2.16, 2.19, 2.21, 2.23, 2.24, 2.25, 2.37	ST-103, ST-11/ET-37, ST-162, ST-174, ST-18, ST-22, ST-23
fIR16	3	3.31, 3.59, 1.648	ST-213
fIR15	3	3.47	ST-461
fIR20	3	3.45	ST-213

Table 4. Association between the 11 most representative fIR types and fHbp variants, fHbp subvariants and clonal complexes.

Analysis of the correlation between fIR type and fHbp coding sequence based on Pearson residuals (Figure 22A and Table 4) revealed that of the 79 fIR types 1, 7, 6, 2, 3 as well as the two new sequence types fir 11 and 13 were mostly associated with fHbp variant 1. Var2 fHbp were positively correlated uniquely to fIR4 and var3 fHbp was found positively associated with fIR types 16, 15, 20 and other less representative sequences. The same analysis carried out on fHbp subvariants highlighted that with the exception of fIR11, 4 and 16, the other fIR types were mostly associated with a specific peptide of fHbp (Figure 22B and Table 4). It is worth noting that fIR6 and fIR13, which share a highly similar intergenic region, are both associated with var1.14 and contrarily all var2 subvariants have the conserved fIR4 type. This suggests that most subvariants will be driven by a specific intergenic sequence associated with them and therefore generally may exhibit distinct and similar levels of expression. For example strains expressing var1.1 and var1.15 will generally express high levels of fHbp as they have fIR1 and fIR7,

respectively. For var2 the large majority of subvariants have the same regulatory sequences and therefore may drive similar fHbp expression levels.

Furthermore, most of the intergenic regions (fIR1, 7, 6, 2, 11, 16, 15 and 20) were found to be associated with a specific clonal complex (CC); whereas, fIR types 3, 13 and 4 were associated with different CCs (Figure 23 and Table 4). Surprisingly, despite the correlation between fIR11 and diverse fHbp peptides, this intergenic region was found mostly in the ST-11/ET-37 complex. These evidences point towards a high frequency of recombination at the level of the fHbp CDS within this CC. Interestingly, strains belonging to diverse intergenic region types, fIR2 and 6, fIR16 and 20, hence expressing different amounts of fHbp were found to be associated to the same CCs, ST-41/44 Lineage 3 and ST-213, respectively. This indicates a lower association between fHbp amounts and CCs. An additional analysis on the correlation between fHbp peptides and CCs in the strains belonging to the 11 most representative fIR types showed that clonal complexes were often associated with one or few peptides of the antigen (Figure 24).

Taken together these data suggest a possible evolution of the *fHbp* intergenic region with its corresponding coding sequence, which often evolved within a specific CC. Conversely, most of the peptides of var2 have evolved to maintain the same regulatory elements for the tuning of fHbp expression. There are exceptions, such as ST-11 and ST-213 where the same promoter (fIR11 for ST-11, and fIR16 and fIR20 for ST-213) drives the expression of multiple subvariants.

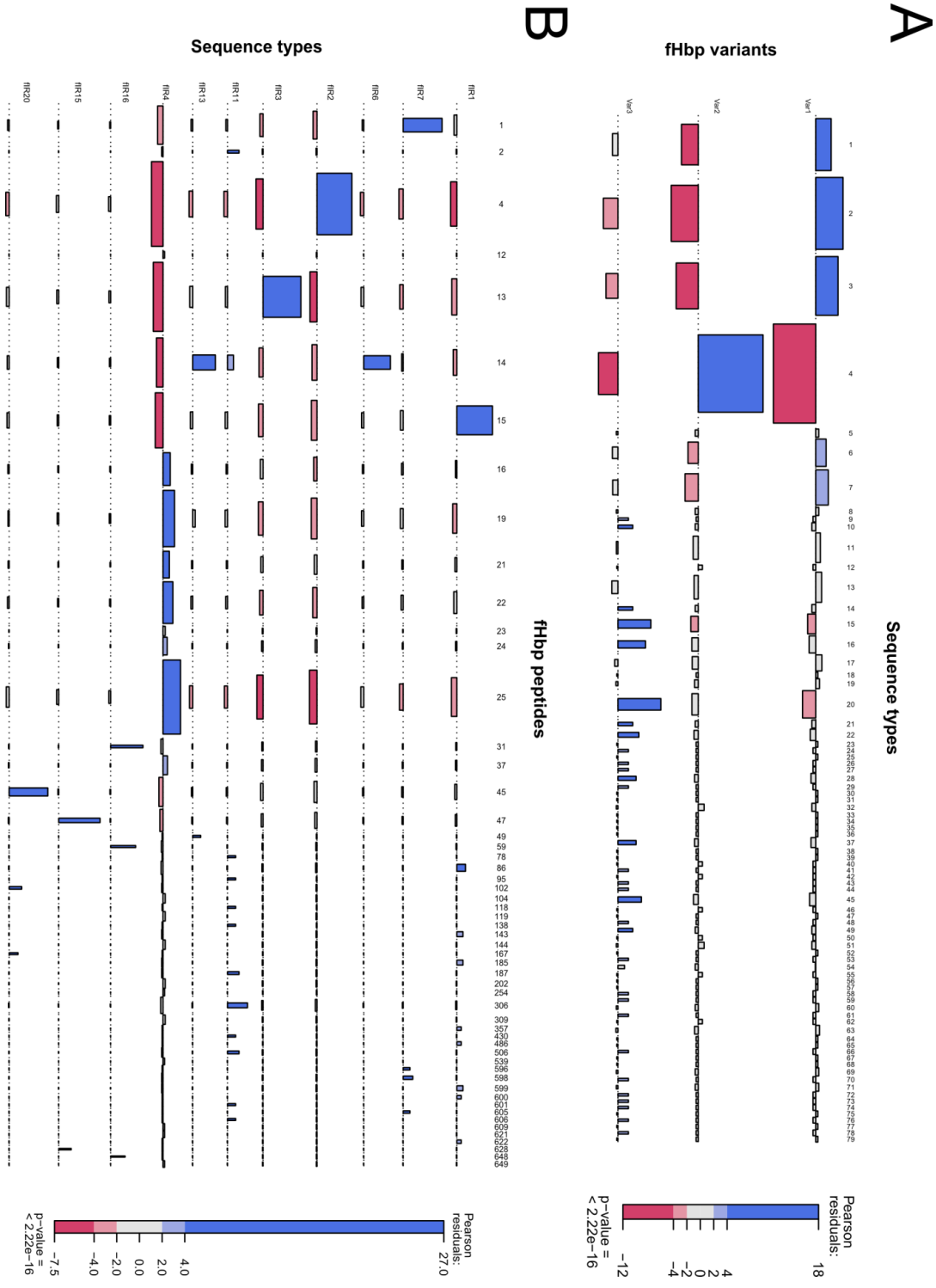

Figure 22. Statistical analysis with Pearson residuals. (**A**) Association between fIR types and fHbp variant. Colors reflect the significance of the association as the bar on the right indicates. Bar width is proportional to the number of intergenic sequences and bar height is proportional to residual of observed versus expected. (**B**) Association between fHbp peptides and fIR types.

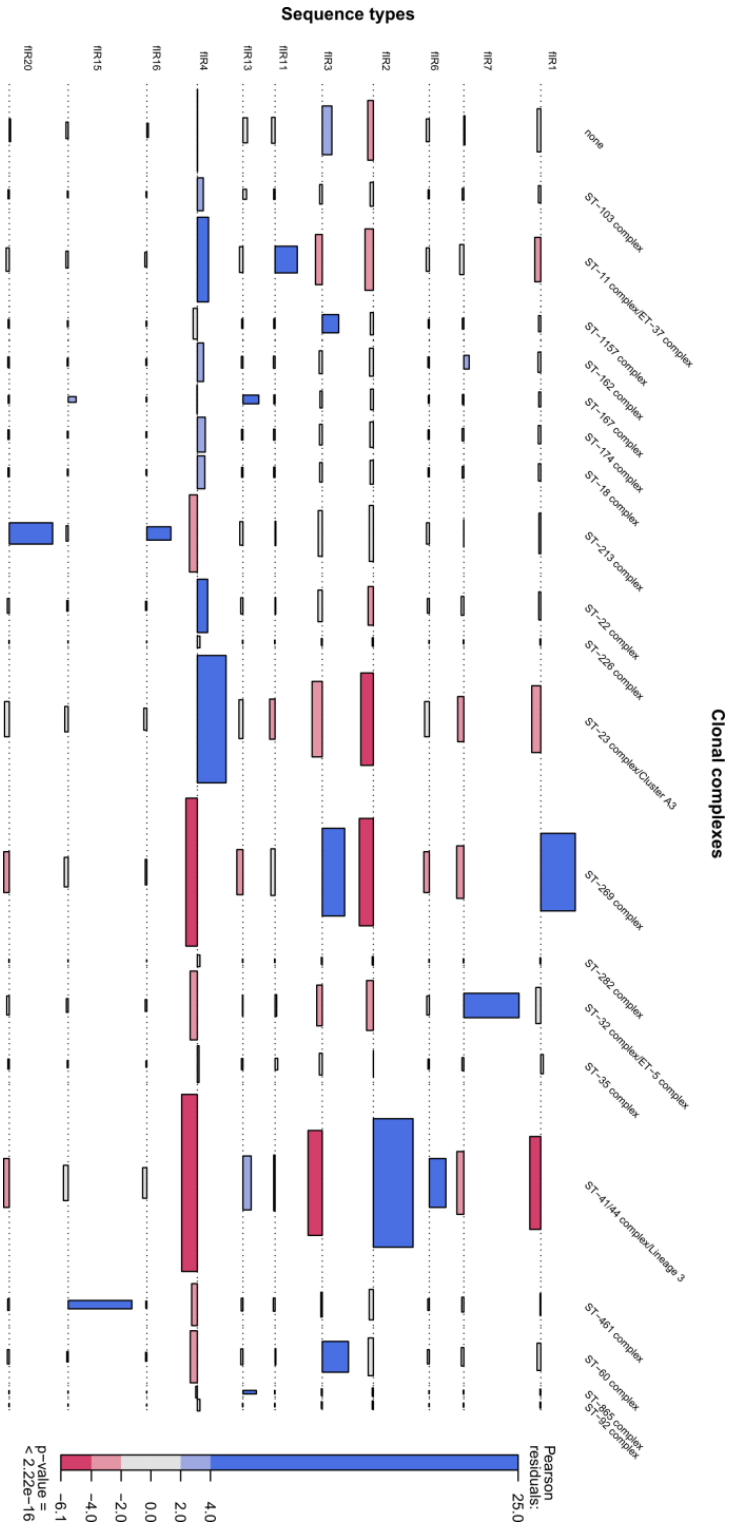

Figure 23. Statistical analysis of the association between clonal complexes and fIR types.

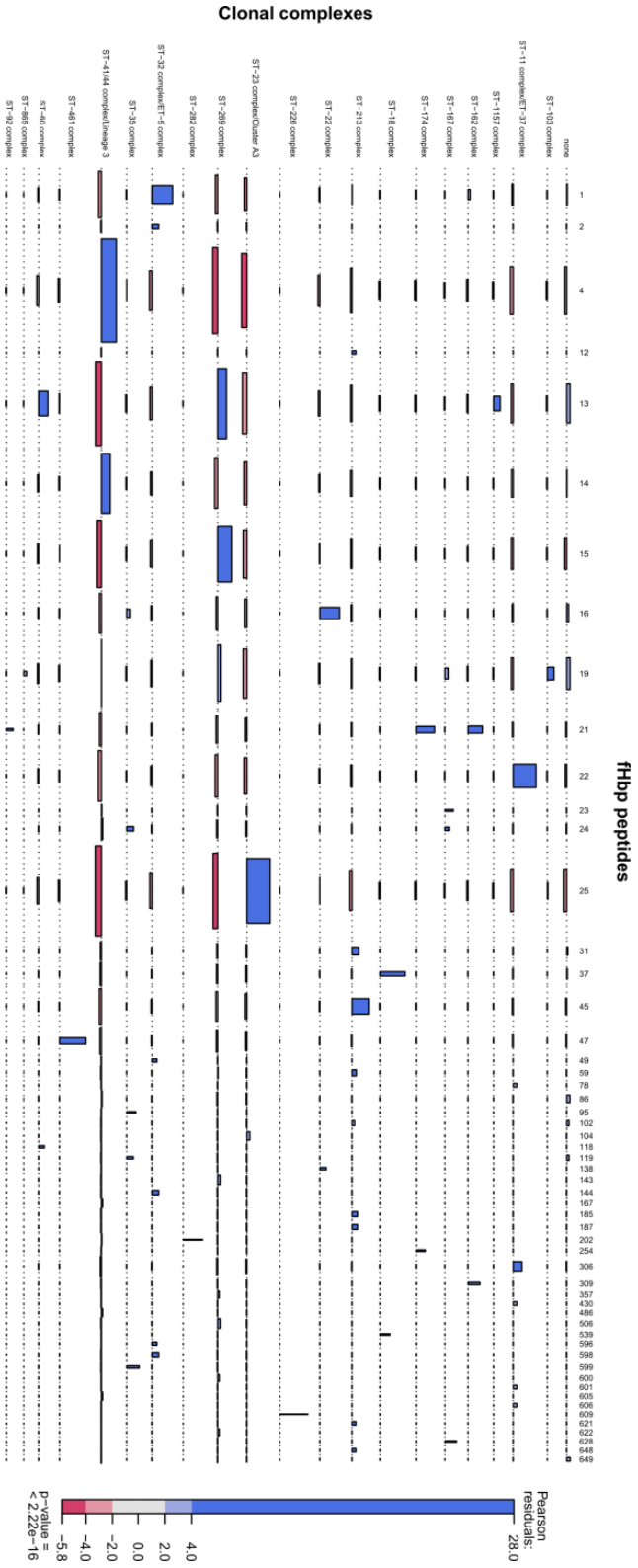

Figure 24. Statistical analysis of the association between fHbp peptides and clonal complexes.

3.10. fIR types in other *Neisseria* species

Factor H binding protein is also present in other species of the genus *Neisseria*, such as the pathogen *N. gonorrhoeae* and the non-pathogenic *N. cinerea* and *N. polysaccharea* [88]. We extracted the DNA sequences of the *fHbp* intergenic region of these strains from public available databases and their alignment allowed the identification of several SNPs (Figure 25). None of the sequences were included in the 11 classes identified for meningococcal strains. The intergenic region of *fHbp* of *N. polysaccharea* ATCC 43768 was similar to fIR16, with the exception of a SNP within the -35 box (TTGGTG) and a shorter spacer; moreover, it was the only one sequence among the panel containing a noncanonical a -10 element (TACCGC). However, often the CDS was frameshifted in different positions (data not shown). Noteworthy, all gonococcal strains conserved the same sequence which contained a 1 nt-deletion within the terminator region, resulting in a ΔG = -24.8. This is in accord with the high conservation of the locus in gonococcus [88]. Rho-independent terminators strength in *N. cinerea* strains was between ΔG = -25.0 and -27.3. Furthermore, two strains, CCUG 27178A and CCUG 346T, contained a SNP within the FNR box, which might impair the binding of the transcriptional regulator. All these suggest that fHbp is expressed at a low/medium level in other *Neisseria spp*. Altogether these findings suggest a different selective pressure on the *fHbp* intergenic region of non-meningococcal strains.

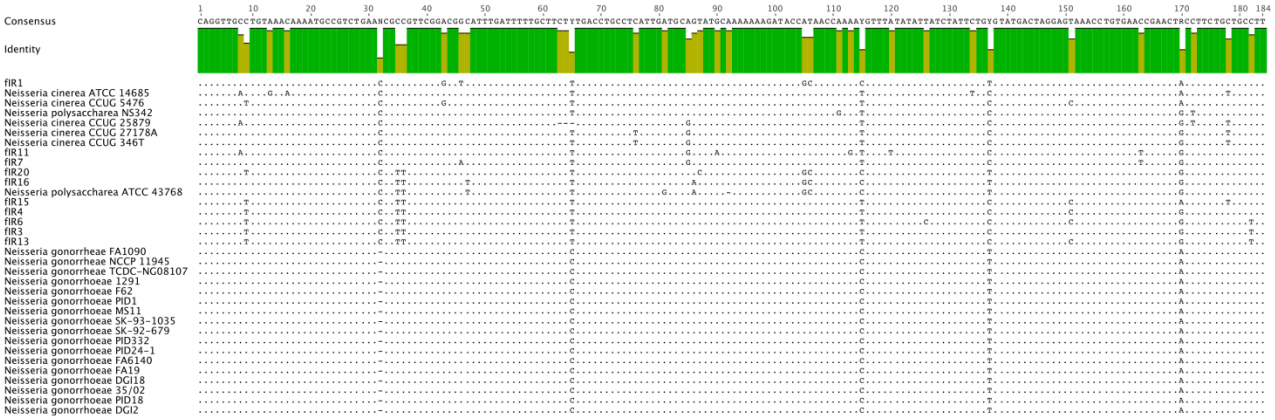

Figure 25. Multiple sequence alignment of the fHbp intergenic region in other *Neisseria* species. DNA sequences of the *fHbp* intergenic regions of *N. cinerea*, *N. polysaccharea* and *N. gonorrhoeae* were aligned together with the sequences of the 11 fIR types identified in *N. meningitidis*. The consensus sequence is at the top of the alignment. Green areas indicate nucleotides with 100% identity, and variable regions are indicated in yellow. Dots represent conserved positions, mismatches are indicated with nucleotides and gaps with "-".

4. Discussion

The meningococcal Factor H binding protein is an important component of two licensed vaccines against MenB: Bexsero®, which contains fHbp var1.1 plus two additional recombinant protein antigens (NadA and NHBA) and an OMV component; and Trumenba®, which contains two fHbp subvariants, each belonging to one of the two subfamilies (var1.55 and var3.45). Expression of fHbp is necessary to achieve complement-mediated bactericidal killing of a given strain by anti-fHbp antibodies and especially in the case of strains carrying fHbp variants distant in sequence from those contained in the vaccine it has been suggested that the level of expression my play a pivotal role [72, 143, 150]. It is known that circulating strains exhibit very different levels of expression of fHbp [72, 73, 85, 90]. In this study we investigate the reasons for the diverse expression levels between circulating meningococcus strains and their implications for vaccine coverage. Previous studies have identified the elements within the intergenic region between *cbbA* and *fHbp* (here referred as *fHbp* intergenic region) responsible for the regulation of expression of this important antigen [90, 95, 98].

Here, we identify the genetic determinants that play a role in control of expression levels, through analysis of the sequence of the *fHbp* intergenic region or fIR sequence type, in 2 strain panels. The first panel includes 105 isolates and is representative of the most common subvariants of fHbp within the three variant groups; we then extend our analysis to a panel of 915 clinical isolates that consists of circulating strains collected in the UK from 2010 to 2012. Bioinformatics analysis of the panel of 915 UK lead to the identification of 79 different fIR sequence types, 11 of which represent roughly 89% of the strains. Nine of these fIR types were also identified in the 105 strain panel and proteomic analysis demonstrated that strains harboring these fIR sequence types gave similar levels of fHbp expression. Therefore, fIR sequence type was found to be associated with tight ranges of fHbp expression in natural strains. Furthermore, generation of isogenic strains, differing only in fIR sequences showed different and distinct levels of expression of fHbp both at the RNA and protein levels and served as proof of concept that the level of fHbp was determined by the specific fIR sequence. As a result, our data suggest that we

can predict the fHbp expression level of a strain which expresses a fIR sequence type that corresponds to one of the 11 analyzed in this study.

Previous studies have suggested that strains harboring var1 fHbp produce higher amounts of fHbp than isolates carrying var2 and var3 [151]. With the use of SRM-MS we were able to measure absolute levels of fHbp in an antibody-independent manner, and therefore not dependent on cross recognition of the variants. The data obtained by SRM-MS [143] reflect the amount of fHbp that is present under standard *in vitro* culturing of the bacterium. This is the result of a tight interplay between gene transcription and mRNA abundance on one hand, and mechanism of post-translational modifications and protein turn over, on the other hand. The significant degree of correlation found between protein amount and sequence of the fHbp intergenic region indicates that gene transcription plays a major role in fHbp expression. In this study we demonstrate that out of the 105 panel analyzed the var1 carrying strains express more fHbp than var2 and 3. Furthermore, we also define that the molecular reasons for this are twofold. Firstly, the var1 strains generally carry fIR sequence types that lead to higher transcription of the fHbp coding sequence downstream, such as fIR1, 7, 6, 2, 3, 11 and 13. In addition, our data show that, in an isogenic background, similar transcript levels of different fHbp subvariants corresponded to less protein amounts of the var2 and var3 with the respect of var1. This evidence is in agreement with Differential Scanning Calorimetry analysis, which indicates significantly lower melting temperatures for the N-terminal domains of var2 and var3 compared to the N-terminus of var1 [147, 148]. Furthermore, while three-dimensional structures of var1 fHbp subvariants could be obtained both by Nuclear Magnetic Resonance and by X-ray crystallography [80, 81, 141, 148, 152], full length structure of var2 has never been determined, and crystallization of full length var3 was only achieved in the presence of hfH as stabilizing factor [148]. Therefore, the coding sequence of fHbp influences either protein stability or its translation efficiency. In order to address this question more experiments would be needed. In summary, var1 strains express more fHbp due to a combination of stability of the protein and association with fIR types leading to higher transcription. Interestingly, var1-intergenic regions were found to be generally associated with hyper-invasive clonal complexes (ST-269, ST-32, ST-11 and ST-41/44). Moreover, by comparing fHbp amounts produced from fIR types 2 and 4, the insertion of the ATR-mobile element [72, 90, 95] that discriminates the two intergenic regions is responsible for a slightly

increase in the expression level of the fIR2 strain. These evidences suggest that alleles of var1 fHbp together with their corresponding intergenic regions have evolved to generally express more protein, hence increasing strains' fitness within the host.

In a previous study the fHbp expression has been reported to be linked to CCs [95], however our data suggest that rather than correlate with any of the conventional classifications such as serotype, clonal complex or sequence type [143], the expression level from strain correlates to and is determined by the intergenic region upstream of *fHbp* and therefore the fIR sequence type. We found that there is a strong association between fIR types and fHbp peptides pointing towards a coevolution of the two sequences, which is in line with their linkage. There were however two exceptions to this general rule. Firstly, it is intriguing that we found the presence of intergenic regions associated with specific clonal complexes that express different fHbp subvariants, which suggests both a high recombination rate of the downstream coding sequence and a selective pressure to maintain the same regulatory elements for fHbp expression. From our analyses this occurs in ST-11 where a range of heterologous subvariants (largely from var1 but also including variants 2 and 3) of fHbp are all expressed from the fIR11 sequence type, and to some extent in ST-213 where subvariants 3.31, 3.59 and 1.648 are expressed from fIR16 sequence type. Interestingly these CCs are associated with hyper-invasive strains causing sporadic epidemics of invasive disease. The clonal complex ST-11, that is most frequently of serogroup C and W, has been provoking outbreaks and epidemics of invasive meningococcal disease worldwide with high mortality rate [153-155]. Recombination involving major outer membrane antigen genes ("antigenic shift") has been linked to increased incidence of meningococcal disease [156]. The elevated levels of disease caused by ST-11 and the historical significance of these hyper-invasive meningococci have made them a priority for whole genome sequencing analyses giving rise to high resolution data which facilitate surveillance and the identification of differences in outbreak strains [153-155]. The emergence of the "Hajj clone" in 2000, which caused an epidemic during the annual Hajj pilgrimage to Mecca in Saudi Arabia [157], was caused by a meningococcal strain that was distinct from other circulating serogroup W135 ST-11 strains. The fHbp locus has been recently identified as one genomic region within the hyper-invasive lineage that undergoes allelic replacement through recombination likely involving donor sequences from meningococci outside ST-11 lineage and commensal *Neisseria* species [153]. Before 2000, most ST-11 strains

carried a variant 2 or 3 variant, while the allele associated with the Hajj clone carries a variant 1 (subvariant 9). The introduction of a novel fHbp antigenic type into an immunologically naïve population may have played a part in the emergence of the Hajj clone. Intriguingly, during these recombination events, our data indicate that the regulation and expression of fHbp is maintained as the fIR11 sequence type is not a target for recombination in this important hyper-invasive clonal complex ST-11.

Secondly, the strong correlation between nearly all subvariants of var2 expressed from diverse clonal complexes (ST-103, ST-11/ET-37, ST-162, ST-174, ST-18, ST-22 and ST-23) and the same low producing-fIR type 4 is a noteworthy observation from this study and sheds light on two aspects. The classification of fHbp variants has been somewhat controversial, and either 3 variants [72] or two subfamilies [73] are considered. As such whether var2 and var3 could be classified as one subfamily is a fundamental molecular epidemiological conundrum. This study shows that apart from the genetic distance of the coding sequences there is a fundamental difference between var2 and var3 with respect to their associated regulatory regions. While almost all variant 2 strains have the fIR4 sequence type, var3 are associated with remarkable diversity at the promoter level and this observation stresses their segregation into two groups [72] rather than a single subfamily [73]. Furthermore, carriage strains have been associated with lower level of expression than invasive strains [158] and var2 expressing strains have been associated more with carriage rather than invasive isolate [159]. Here we observe that var2 are generally lowly expressed due possibly to a combination of protein turnover and a conserved low expressing fIR sequence type (fIR4). This is in line with both the previous observations in which relative lower fHbp expression and a higher association of var2 was observed in carriage strains with the respect of invasive isolates [158]. In the strain panels analyzed, all isolates were from disease cases, therefore, indeed, it would be interesting to analyze the *fHbp* intergenic region sequences within a panel of carriage-associated isolates.

The key role of fHbp in *Neisseria meningitidis* [74, 78] in binding fH and therefore in resistance of meningococcus to complement is highlighted by the fact that nearly all strains express this antigen [84, 85, 90, 91]. The relatively small number of fIR types which control its expression might suggest a high selective pressure in the locus in maintaining certain expression levels. fHbp in other species of *Neisseria* is less essential. Indeed, frameshifts in different positions of its

coding sequence were identified in isolates of *N. cinerea* and *N. polysaccharea* [88] and the gonococcal fHbp (Gfhp) is not surface exposed, due to a frameshift in the signal peptide sequence [89]. Apart from gonococcus, where the *gfhp* gene [89, 160] and fIR region are highly conserved, the *fHbp* intergenic region of the other species of the genus *Neisseria* in which the gene is present [88] contains several polymorphisms and do not clearly cluster. Taken together these evidences suggest the absence of a significant selective pressure on the *fHbp* region in these species. Even though Gfhp is not surface exposed [89], the presence of a 1-nt deletion within the Rho-independent terminator region would have a positive impact on its expression level and the protein might have a different role in *N. gonorrhoeae.*

The polymorphisms segregating the fIR sequence type are present in previously identified regulatory elements including transcriptional elements, such as the Rho-independent terminator from the *cbbA* upstream gene and the FNR-regulated *fHbp* promoter as well as post-translational elements such as an RNA structure within the 5'UTR and 5' coding sequence hypothesized to be involved in thermosensing and control [90, 98]. A deeper molecular analysis of the *fHbp* intergenic regions revealed a major contribution of the Rho-independent terminator and the -10 box on the antigen expression levels. While the presence of a bicistronic transcript deriving from the upstream P_{cbbA} was observed for MC58 [90] and more in general for isolates belonging to ST-32 [95], here we demonstrate that, irrespective of the CC, the longer transcript is generated uniquely when polymorphisms within the stem region of the terminator affect its pairing and decrease its strength, hence allowing read-through of the RNA polymerase. Therefore, even if expression from *cbbA* is generally stable [90], the promoter driving expression of *cbbA* would affect *fHbp* levels only in strains having weak terminators. A canonical -10 element is composed of A and T (TATAAT); however, the most frequent -10 hexamer for the fHbp promoter contains two Cs (TACCAT). Nonetheless, several strains contain a -10 sequence where only two out of six nucleotides are A or T (TACCGC). This high GC content would reduce recognition by the holo RNA polymerase and possibly fork opening, hence resulting in lower transcription rates as we have demonstrated using the isogenic mutant strains. Interestingly, only three fIR types contained the TACCGC allele and two of them were associated with weak or medium terminators that would counterbalance its negative effects on fHbp expression. Overall, four groups of expression levels were detected (fIR1/7 > fIR6/16 > fIR2/3/15 > fIR4/20, groups 1, 2, 3 and 4, respectively)

and the molecular mechanisms leading to these distinct levels were elucidated. Furthermore these expression levels directly resulted in four groupings of distinct susceptibility to anti-fHbp antibodies bactericidal killing. Notably, with the substitution of a T with a C, from (TTGATG) to (TTGACG), identified in only two out of 915 strains, the -35 box of fHbp would better resemble a canonical one (TTGACA); this could explain the massive increase of protein amounts produced that we observe in our experiments. This polymorphism, although very rare (0.2% frequency), occurred linked to var1 in two strains identified in the UK collection and to var2 in M08-0240104 and the three strains belong to three different CCs (ST-269, ST-41/44 and ST-35, respectively). It would be interesting to track when these polymorphisms arose, and whether strains with this SNP are isolated outside of the UK.

During colonization and infection *N. meningitidis* encounters different environments and is exposed to components of the immune system. Previous studies demonstrated the role of fHbp in binding the human Factor H allowing the meningococcus to evade complement-mediated killing in blood. Furthermore, the presence of complement components on the mucosal surface suggests that the advantages conferred to the bacterium by fHbp might be extended to the initial stages of colonization and invasion. As *N. meningitidis* moves from the upper respiratory tract to the blood and cerebrospinal fluid, the bacteria would be exposed to highly different oxygen pressures. Under oxygen limiting conditions the transcriptional regulator FNR binds the dedicated *fHbp* promoter and increases its expression [90]. In line with this, excluding fIR1, even if at different extents, there is a trend for increases in *fHbp* expression under limited oxygen in all of the intergenic region mutants tested. In addition, we show that in MC58 no significance differences are observed in *cbbA* expression levels between the two conditions. As suggested from Oriente *et al*., the cumulative effect of this regulation might not always arise at the protein level in the strains where read through is observed. This may be the case for fIR1 where we measured higher steady state level of both *cbbA* and bicistronic transcripts than in the other strains, suggesting that the overall regulation of fHbp through the dedicated promoter is overridden by the higher quantities of read-through from the *cbbA* driven transcript. In a different work Sanders and collaborators showed a decrease of *fHbp* transcription levels in iron-depleted conditions, with the exception of strains belonging to ST-32, where *fHbp* levels increased. However, data in this study indicated that fHbp levels are not affected or only slightly by iron depletion in the presence of a

weak or medium Rho-independent terminator; whereas, the effects are accentuated for the other intergenic region types. The differential regulation observed is dependent on the presence of the bicistronic transcript and not specifically on the clonal complex as fIR types 1, 7 and 16 are not limited to ST-32. It is plausible therefore that the fIR types with read-through (1, 7 and 16) and without (all the rest) may be differentially regulated depending whether the cumulative effects of the bicistronic transcript steady states out weight regulation of the *fHbp* dedicated promoter.

The intriguing possibility that fHbp might be thermoregulated would increase the fitness of the bacteria when facing an inflammation status or when entering the bloodstream (37°C) from the nasopharynx (30-32°C). However, in our conditions we do not observe any protein level differences between 30 and 37°C.

In agreement with the observation that the MC58 strain is dependent on fHbp to survive within human serum [70, 78], we show that the ability of the strain to evade the humoral immune response is dependent on surface exposed-fHbp amounts and that the different intergenic region types identified respond differently to the stimulus. Indeed, fIR types associated with high expression of the protein (fIR1, 7 and 16) determine higher percentage of survival and fIR types linked to low expressing strains (fIR3, 4 and 15) are generally killed, with the fascinating exception of fIR20, which express low quantity of fHbp *in vitro*, but survives at levels comparable to high-medium expressors. The peculiarity of this fIR type is that it contains the alternative -10 element (TACCGC) in the absence of a bicistronic transcript. Since no significant regulation under oxygen and iron limitation were observed for fHbp under the control of this intergenic region, it is not impossible that this sequence might undergo a specific induction/regulation under incubation with human serum which would explain this surprising resistant phenotype. However, the model system of isogenic strains employed here for the experiments did not take into account the different protein stability of the three fHbp variants and their respective affinity with hfH which would affect survival in human serum. In summary, we have the first observations that fIR sequence types not only determine distinct expression levels, but may infer different regulatory control in response to environmental changes/stimuli. The different expression levels determined by both oxygen and iron availability might have implications on the outcome of biological assays that evaluate vaccine potency and coverage, such as SBA and MATS. In fact, the assays might under or overestimate the protection offered by

anti-fHbp antibodies, depending on the *in vitro* conditions used to grow the meningococcal isolates to evaluate. A full understanding of the mechanisms and extent of differential regulation between fIR types is important.

It has previously been shown that the ability of meningococcal strains to survive in human serum is the result of a multiplicity of factors. In fact, while the deletion of the *fHbp* gene in MC58 results in a strain that is readily killed in human serum [70, 78, 161], the same deletion does not affect the capacity of other strains to survive in human serum, indicating the critical role played by other factors, like NspA and PorB2/PorB3, in these strains [36, 37, 70, 162-164]. Therefore, strains which display low fHbp expression might rely on other mechanisms such as those listed for the evasion of the immune system.

The serum bactericidal assay (SBA) is considered as correlate of protection for meningococcal vaccines [135]. It evaluates the capability of antibodies to mediate killing of *N. meningitidis* strains in the presence of a complement source. Here we show that fHbp amounts exposed on the surface of recombinant strains were correlated with SBA titers. Also, not included in this study we have reported that fHbp levels determined by natural promoters and assessed after an *in vitro* growth are correlated with susceptibility of the bacteria to complement-mediated killing. In human serum fH and factor H-related protein 3 (FHR3) compete with antibodies for fHbp binding and therefore the number of molecules required for positive SBA in human serum as complement source may vary as a result of individual fH and FHR3 levels. Furthermore, as for the human serum survival assay, the use of a model system might not take into account different affinity of the three fHbp variants to hfH and from a vaccine standpoint, it would be necessary to consider the distance of the fHbp variant sequence from those contained in the vaccine.

Altogether these evidences point towards the use of the nucleotide sequence of the *fHbp* intergenic region as a predictive tool for (i) the amount of antigen produced by any strain, even the ones not culturable under lab conditions; (ii) the changes in fHbp expression levels in response to exogenous stimuli. Hence, together with the coding sequence, it would be possible to evaluate the fHbp-dependent vaccine coverage, as an *in silico* fHbp-Meningococcal Antigen Typing System (MATS). Therefore, an implementation of the fIR type classification within the PubMLST would be beneficial and will be implemented. Using this fIR-based analysis in the circulating strains in the UK over a three year period, 13.2% (fIR1 and 7, containing largely var1)

are predicted to express high levels of fHbp, 35.5% (fIR4 and 20, largely var2 and some var3) low levels, 3.5% medium high (fIR6 and 16, containing var1 and var3), and 36.6% (fIR2, 3, 11, 13 and 15, containing largely var1 and some var3) medium low levels of fHbp. Our data shows that bactericidal fHbp-antibody immune responses, which is a correlate of protection of a vaccine, directly relates to these diverse levels of expression. Therefore, vaccines containing only fHbp may not protect efficiently against strains expressing low amounts of fHbp, which represent a large proportion, and this subgroup can be potentially identified by sequencing. Our analysis supports the use of a multi-component vaccine with multiple protective antigens.

5. Materials and Methods

5.1 Bacterial strains and culture conditions

Neisseria meningitidis strains used in this study are reported in Table 5. Strains were routinely cultured overnight on Gonococcus (GC) agar medium (Difco) with Kellogg's supplement I [165] at 37°C in an atmosphere of 5% CO_2. Liquid cultures were grown under the same conditions in GC with Kellogg's supplement I or in Mueller Hinton (MH) broth plus 0.25% glucose. When required, erythromycin (5 μg/ml), chloramphenicol (5 μg/ml) or isopropylβ-D-1-thiogalactopyranoside (IPTG) (1 mM) (Sigma) were added to culture media at the indicated final concentrations.

To test iron starvation *N. meningitidis* strains were grown till OD_{600} of 0.5. Cultures were then split and treated either with 250 μM 2,2-dipyridyl (Sigma) dissolved in ethanol or with ethanol (not treated condition) for 30 min. Oxygen limitation was tested as already described [90].

Escherichia coli DH5α [166] and HK100 [167] strains were grown in Luria-Bertani medium, and when required, ampicillin or chloramphenicol were added to achieve a final concentration of 100 μg/ml and 10 μg/ml, respectively.

5.2 Construction of mutant and complementation strains

DNA manipulations were carried out routinely as described for standard laboratory methods [168]. The kanamycin resistance cassette of the plasmid pBS-c741 wt KanR used in [90] for the complementation of the *fHbp* gene in the MC58 deletion mutant was substituted with a chloramphenicol-resistance cassette. A mutation present in the coding sequence of the gene was restored by site-directed mutagenesis using primers SNP3PfHbp-F/R (pBS-c741 wt CmR). This plasmid was used as a template for the generation of a series of mutants of the regulatory elements of the intergenic region of *fHbp*; site-directed mutagenesis was carried out using primers TACCAC_TACCAT-F/R (TACCAT -10 box), TACCAC_TACCGC-F/R (TACCGC -10 box), GACGACA_GACGGCA-F/R (from weak to strong terminator), CGGTATG_CAGTATG-F/R (spacer 1 allele), CAGTATG_CAATATG-F/R (spacer 2 allele, using spacer 1 as template),

CAGTATG_CAGCATG-F/R (spacer 3 allele, using spacer 1 as template), and TTGATG_TTGACG-F/R (TTGACG -35 box) (Table 7). The generation of the mutants where the expression of fHbp var1.1 was under the control of the different fIR types identified was performed by polymerase incomplete primer extension (PIPE) method [167] using primers vPCRpBSc741-F/R for the amplification of the backbone plasmid containing the *fHbp* var1.1 gene and primers iPCRprom-F/R for the amplification of the intergenic regions. The complementation of the IPTG-inducible fHbp in the region between the converging ORF NMB1428 and NMB1429 was carried out using the *fHbp* gene amplified (using primers 741-F2/R2 for var1.1, EP1For1.1 and EP5RV1.4 for var1.14, EP2For1.4 and EP6RV2.1 for var2.16 and var2.25, EP1For1.1 and EP6RV2.1 for var3.28 [161], 2.21 fw/rev for var2.21, 3.45 fw and 3.45/47 rev for var3.45, and 3.47 fw and 3.45/47 rev for var3.47) and cloned as a NdeI-NsiI fragment into the $pComP_{IND}$ plasmid [169]. All the PCR amplifications were performed using the KAPA Hi-FI polymerase (KAPA Biosystem) and digesting the DNA template with the DpnI enzyme when required. The correct nucleotide sequence of each plasmid was confirmed by DNA sequencing (Table 6). The plasmids were linearized and used for the transformation of the MC58 Δ*fHbp* strain to create complementation mutants (Table 5) that were selected on GC agar with chloramphenicol. All transformants were verified both by Western blot and by PCR analysis for the correct insertion by a double homologous recombination event (pairs of primers pRTNM_nmb1869U-F/pRTfHbpU.R and CmR-down/complcheck-dsGENOME-R, COM-C-Fw/CM-UP-C and pRTfHbpU.F/COM-C-Rev were used for the *in locus* or *ex locus* complementation, respectively).

5.3 RNA isolation and cDNA preparation

Bacterial cultures were grown in liquid medium to an OD_{600} of 0.5-0.6 and then added to 3 ml of frozen medium to bring the temperature immediately to 4ºC. Cells were harvested by centrifugation at 3400 x g for 10 minutes. Total RNA was isolated using the RNeasy Mini kit (Qiagen) following manufacturer's instructions. RNA samples were incubated with RQ1 RNase-Free DNase (Promega) for an hour at 37°C and purified with the RNeasy Mini kit. 2 µg of total RNA were reverse-transcribed using random hexamer primers and SuperScript® II RT (ThermoFisher) following manufacturer's instructions.

5.4 Quantitative real-time PCR (qRT-PCR) experiments

Quantitative real time-PCR was performed with triplicate biological samples in a 25 µl reaction mixture containing 2.5 ng of cDNA, 2X Platinum® SYBR® Green qPCR SuperMix-UDG with Rox (ThermoFisher) and 0.4 µM of gene-specific primers (Table 7). Amplification and detection of specific products were performed with a Mx3000P Real-Time PCR system (Stratagene) using the following procedure: 95ºC for 10 min, followed by 40 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s then ending with a dissociation curve analysis. The *16S RNA* gene was used as the endogenous reference control and the relative transcript change was determined using the $2^{-\Delta\Delta Ct}$ relative quantification method [170]. Two-way ANOVA was used to calculate statistical significance ($p < 0.05$).

5.5 Serum Bactericidal Activity (SBA) analysis

Serum bactericidal activity against *N. meningitidis* strains was evaluated as previously described [171] with pooled baby rabbit serum (Cederlane). Bacteria were grown in Mueller Hinton broth (MH), plus 0.25% glucose for approximately 1.5 h at 37°C with shaking until early log phase (OD_{600} of ~0.25) and then diluted in Dulbecco's saline phosphate buffer (Sigma) with 0.1% (w/v) glucose and 1% (w/v) BSA (Bovine Serum Albumin) to approximately 10^5 CFU/ml. Serum bactericidal titers were defined as the serum dilution resulting in a 50% decrease in the CFU/ml after 60 min of incubation of bacteria with the reaction mixture, compared to the control CFU/ml at time zero. Typically, bacteria incubated with the negative-control antibody in the presence of complement showed a 150 to 200% increase in CFU/ml during the 60-min incubation.

5.6 Western blot analysis

Strains grown overnight on agar plates were resuspended in PBS to an OD_{600} of 0.80. One milliliter of the resuspension was centrifuged for 5 min at 15000 x g and the pellet was resuspended in 160 µl of SDS loading buffer (50 mM Tris-HCl [pH 6.8], 2.5% SDS, 0.1% bromophenol blue, 10% glycerol, 5% β-mercaptoethanol, 50 mM DTT) [90]. In the case of liquid cultures, strains were grown till an OD_{600} of 0.50 and one milliliter of the culture was pelleted

and resuspended in 100 µl of SDS loading buffer. Protein extracts were separated by SDS-PAGE on NuPAGE® Novex® 4-12% Bis-Tris Protein Gels in MES 1X (Life Technologies) and then transferred to nitrocellulose membranes. Membranes were blocked overnight at 4°C with PBS + 0.05% Tween 20 (Sigma) and 10% powdered milk (Sigma). A mouse anti-fHbp var1.1 serum was diluted (1:2000) in PBS + 0.05% Tween 20 and 3% powdered milk and incubated for 1 h with the membrane. A horseradish peroxidase-conjugated anti-mouse IgG antibody and the Western Lightning ECL (Perkin Elmer) were used according to the manufacturer's instructions.

5.7 Fluorescence Activate Cell Sorting (FACS) analysis of fHbp expression

The ability of mouse polyclonal anti-fHbp var1.1 serum to bind to the surface of meningococci wild type MC58 strain, MC58 Δ*fHbp* and MC58 Δ*fHbp* complemented with *fHbp* var1.1 under the control of an IPTG-inducible promoter was determined using a FACScan flow cytometer using a 1:400 dilution of mouse polyclonal antiserum anti-fHbp var1.1. Primary antibody binding was detected using an anti-mouse (whole-molecule) FITC-conjugated antibody (Sigma) at a 1:100 dilution.

5.8 Survival experiments in human serum

Strains MC58, MC58 Δ*fHbp* and MC58 Δ*fHbp* complemented with *fHbp* var1.1 under the control of an IPTG-inducible promoter were grown in the same conditions as for the SBA assay, till an OD_{600} of 0.25. Bacteria were then diluted to ~10^4 CFU/ml in a total volume of 200 µl GC liquid medium in a 96-well plate. The assay was started by the addition of 10 µl of the bacterial suspension to 190 µl of 40% human serum (Sigma) diluted in HBSS++ (Sigma) in a 96-well plate. Increasing concentrations of IPTG were also added in order to maintain the expression of the protein over time. Samples were incubated at 37°C and 5% CO_2, 180 rpm. At various time points (30, 60, 120 and 180 min), an aliquot of each sample was plated in serial dilutions onto MH agar to determine the number of viable bacteria and incubated overnight at 37°C and 5% CO_2. Experiments were performed in duplicate. The diluted 200 µl culture in GC was used as growth control in bacterial rich medium. For isogenic recombinant strains were the same fHbp

var1.1 was under the control of the different fHbp intergenic region types, bacteria were diluted to ~10^4 CFU/ml and 10 µl of the bacterial suspension were added to 190 µl of 60% human serum (Sigma) in HBSS++ (Sigma). Samples were taken at various time points (60, 120 and 180 min). Experiments were performed in triplicate.

5.9 Amplification and sequencing of the *fHbp* intergenic region

Chromosomal DNA used as template for the amplification of the intergenic region between *cbbA* and *fHbp* was prepared boiling 50 µl of bacterial resuspension in water. AccuPrime Taq DNA Polymerase System (Life Technologies) and the primers 1869-2F and fHbp_32 (Table 7) were used for the PCR amplifications (35 cycles, 94°C for 30 s, 57°C for 30 s, 68°C for 1 min and 30 s). PCR products were purified with the Agencourt AMPure XP (Beckman Coulter). Sequences were performed using an ABI 377 automatic sequencer (Applied Biosystems) and the primers used were 1869-2F, fHbp_A1 and fHbp_32 (when required also IS-seq-fHbp-F1, F2, F3 and R1 were used). Sequence analysis was performed using the software ContigExpress (Vector NTI).

5.10 Bioinformatics analyses

5.10.1. Phylogenetic analysis

The multiple sequence alignment of the 103 sequenced intergenic loci was performed with MUSCLE (v 3.6). The phylogenetic tree was computed using *phangorn* R package [172], applying *dist.ml* distance modeling function, considering the insertion symbol '-' as valid character, and then applying the UPGMA tree reconstruction method. The multiple sequence alignment of the 915 sequences downloaded from the BigsDB (http://pubmlst.org/software/database/bigsdb/) [149] was performed using the MUSCLE algorithm incorporated within the Geneious software (Biomatters) [173]. The matrix of pairwise distances was computed using *seqinr* R package [174], applying *dist.alignment* distance modeling function and considering gaps in the identity measure (gap = 1). The phylogenetic tree was reconstructed using the Neighbor joining method.

5.10.2. Rho-independent terminators analysis

The strength and secondary structure of the Rho-independent terminators were calculated using the online application FindTerm [145] and setting the energy threshold value as -10. The schematic representations of the terminators were prepared using the Visualization Applet for RNA (VARNA) [146].

5.10.3. fIR types identification

A perl (Practical Extraction and Report Language) script was created to assign an identification number, the fHbp intergenic region (fIR) type, to each unique sequence of the multiple sequence alignment of the intergenic regions downloaded from the BigsDB. Results of this analysis were linked to the metadata of the corresponding strains present in the PubMLST.

5.10.4. Statistical analyses

Statistical analyses on the association between fIR types and fHbp subvariants or clonal complexes were carried out under the R environment. The Pearson residuals [(observed – expected) / sqrt(expected)] were calculated using the *chisq.test* function, which performs chi-squared contingency table tests and goodness-of-fit tests. Visual representation of the analysis was performed using the function *assoc* of the *vcd* R package [175].

5.11 Selected Reaction Monitoring-Mass Spectroscopy analysis

5.11.1. Sample Digestion for SRM-MS.

Bacteria were collected from agar plates and resuspended in 10 ml of PBS to an OD_{600} of 0.8. Bacterial cells were pelleted by centrifugation at 3,000 × g for 15 min and lysed by boiling for 15 min in 500 µl of 5% (wt/vol) SDS, 100 mM Tris·HCl, pH 8, 50 mM DTT, and protease inhibitor mixture (Sigma). The protein concentration was determined using the 2D QuanKit (GE Healthcare) and the samples were stored at -20 °C until their use. Trypsin digestion of lysed bacterial samples was performed in duplicate using a filter-aided sample preparation protocol.

The efficacy of the digestion was checked by SDS/PAGE and by assessing the number of missed cleavages inferior to 10% of identified peptide by LC-MS/MS as described above.

5.11.2. SRM-MS Analysis.

SRM analysis was performed on a TQ Xevo triple quadrupole mass spectrometer (Waters) equipped with an ESI source (Waters). Chromatographic separations of peptides were performed on an Acquity LC system (Waters) equipped with a 1- × 150-mm 1.7 µm CSA C18 column (Waters) by a 10-min linear gradient of 5–40% acetonitrile in water, containing 0.1% formic acid, with a flow rate of 80 µl/min. Both Q1 and Q3 were set at unit resolution (FWHM 0.7 Da). A spray voltage of 1,700 V was used with a heated ion transfer setting of 270 °C for desolvation. Data were acquired using MassLynx software (version 2.1.0; Waters). The dwell time was set to 30 ms and the scan width to 0.02 m/z. The peak area quantification was determined with TargetLynx software (version 1.0.0.1; Waters) after confirming the coelution of all transitions for each peptide and following the best practices reported in Carr *et al*. [176].

5.11.3. PTP dose-range linearity responses and fHbp quantification.

The dose-range linearity response of the selected PTPs was assessed in a lysed bacterial sample prepared from MC58 Δ*fHbp* strain used as reference background to take into account the matrix effect. For fHbp quantification, labeled PTPs (final concentration 20 fmol/µl) and non-labeled PTPs (final concentration from 1.9 to 300 fmol/µl) were spiked in 100 µg of total cell lysate prior to trypsin digestion, and SRM experiments were performed in triplicate, injecting 20 µg of lysate onto the column for LC-SRM analysis. For each PTP, concentrations were plotted as ratio of peak area light (variable)/peak area heavy (constant) and the fitted curve was used to deduce the concentration of selected PTP. The LLOQ for each PTP was set as the lowest concentration point on the fitted curve with an accuracy deviation ≤20%. The fHbp concentrations were reported in picograms per microgram of total protein extract, considering for all proteins the molecular mass of the fHbp var1.1.

Table 5. Strains used in this study.

Name	Description	Antibiotic resistance	Reference
MC58	*Neisseria meningitidis* laboratory-adapted reference strain		Tettelin *et al*., Science 2000
MC58 Δ*fHbp*	MC58 derivative, lacking *fHbp* gene	Erythromycin	Masignani *et al*., J Exp Med 2003
MC58 c-*fHbp* (fIR7)	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and its upstream intergenic region (fIR7)	Chloramphenicol	This study
MC58 c-*fHbp* fIR1	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and the upstream fIR1 intergenic region type	Chloramphenicol	This study
MC58 c-*fHbp* fIR2	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and the upstream fIR2 intergenic region type	Chloramphenicol	This study
MC58 c-*fHbp* fIR3	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and the upstream fIR3 intergenic region type	Chloramphenicol	This study
MC58 c-*fHbp* fIR4	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and the upstream fIR4 intergenic region type	Chloramphenicol	This study
MC58 c-*fHbp* fIR6	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and the upstream fIR6 intergenic region type	Chloramphenicol	This study
MC58 c-*fHbp* fIR11	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and the upstream fIR11 intergenic region type	Chloramphenicol	This study
MC58 c-*fHbp* fIR13	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and the upstream fIR13 intergenic region type	Chloramphenicol	This study
MC58 c-*fHbp* fIR15	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and the upstream fIR15 intergenic region type	Chloramphenicol	This study
MC58 c-*fHbp* fIR16	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and the upstream fIR16 intergenic region type	Chloramphenicol	This study
MC58 c-*fHbp* fIR20	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and the upstream fIR20 intergenic region type	Chloramphenicol	This study
MC58 Δ*fHbp* c-*fHbp* var1.1	MC58 derivative, lacking *fHbp* gene, with a copy of *fHbp* var1.1 reintroduced out of locus under the control of an IPTG-inducible P_{TAC} promoter	Chloramphenicol	Biagini *et al*., PNAS 2016
MC58 Δ*fHbp* c-*fHbp* var1.14	MC58 derivative, lacking *fHbp* gene, with a copy of *fHbp* var1.14 reintroduced out of locus under the control of an IPTG-inducible P_{TAC} promoter	Chloramphenicol	This study
MC58 Δ*fHbp* c-*fHbp* var2.16	MC58 derivative, lacking *fHbp* gene, with a copy of *fHbp* var2.16 reintroduced out of locus under the control of an IPTG-inducible P_{TAC} promoter	Chloramphenicol	This study
MC58 Δ*fHbp* c-*fHbp* var2.21	MC58 derivative, lacking *fHbp* gene, with a copy of *fHbp* var2.21 reintroduced out of locus under the control of an IPTG-inducible P_{TAC} promoter	Chloramphenicol	This study
MC58 Δ*fHbp* c-*fHbp* var2.25	MC58 derivative, lacking *fHbp* gene, with a copy of *fHbp* var2.25 reintroduced out of locus under the control of an IPTG-inducible P_{TAC} promoter	Chloramphenicol	This study
MC58 Δ*fHbp* c-*fHbp* var3.28	MC58 derivative, lacking *fHbp* gene, with a copy of *fHbp* var3.28 reintroduced out of locus under the control of an IPTG-inducible P_{TAC} promoter	Chloramphenicol	This study
MC58 Δ*fHbp* c-*fHbp* var3.45	MC58 derivative, lacking *fHbp* gene, with a copy of *fHbp* var3.45 reintroduced out of locus under the control of an IPTG-inducible P_{TAC} promoter	Chloramphenicol	This study
MC58 Δ*fHbp* c-*fHbp* var3.47	MC58 derivative, lacking *fHbp* gene, with a copy of *fHbp* var3.47 reintroduced out of locus under the control of an IPTG-inducible P_{TAC} promoter	Chloramphenicol	This study
MC58 c-*fHbp* -10 box (TACCGC)	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and its upstream intergenic region (fIR7) mutated in the -10 box: TACCGC	Chloramphenicol	This study
MC58 c-*fHbp* term -27	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and its upstream intergenic region (fIR7) mutated in the terminator: strong	Chloramphenicol	This study
MC58 c-*fHbp* term -27, -10 box (TACCGC)	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and its upstream intergenic region (fIR7) mutated in the terminator: strong; and in the -10 box: TACCGC	Chloramphenicol	This study
MC58 c-*fHbp* term -27, spacer1	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and its upstream intergenic region (fIR7) mutated in the terminator: strong; spacer: CAGTATGCAAAAAAAGA	Chloramphenicol	This study
MC58 c-*fHbp* term -27, spacer2	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and its upstream intergenic region (fIR7) mutated in the terminator: strong; spacer: CAATATGCAAAAAAAGA	Chloramphenicol	This study
MC58 c-*fHbp* term -27, spacer3	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and its upstream intergenic region (fIR7) mutated in the terminator: strong; spacer: CAGCATGCAAAAAAAGA	Chloramphenicol	This study
MC58 c-*fHbp* term -27, -35 box (TTGACG)	MC58 derivative, complemented *in locus* with the wt *fHbp* gene and its upstream intergenic region (fIR7) mutated in the terminator: strong; and in the -35 box: TTGACG	Chloramphenicol	This study

Table 6. Plasmids used in this study.

Name	Description	Antibiotic resistance	Reference
pBluescript (pBS)	Cloning vector	Ampicillin	Novagen
pBS-UD741	pBS containing the flanking region of *fHbp* with a SmaI site in the middle	Ampicillin	Masignani *et al*., J Exp Med 2003
pBS-741 EryR	pBS-UD741 derivative in which a Ery resistance cassette was cloned as a SmaI fragment between flanking regions	Ampicillin, Erythromycin	Masignani *et al*., J Exp Med 2003
pBS-c741 wt KanR	Plasmid for the *in locus* complementation of the *fHbp* gene including its upstream promoter region. Downstream of *fHbp* is cloned a Kan resistance cassette	Ampicillin, Kanamycin	Oriente *et al*., J Bacteriol 2010
pBS-c741 wt CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including its upstream promoter region (fIR7). Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 fIR1 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including the upstream fIR1 sequence type. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 fIR2 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including the upstream fIR2 sequence type. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 fIR3 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including the upstream fIR3 sequence type. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 fIR4 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including the upstream fIR4 sequence type. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 fIR6 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including the upstream fIR6 sequence type. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 fIR11 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including the upstream fIR11 sequence type. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 fIR13 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including the upstream fIR13 sequence type. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 fIR15 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including the upstream fIR15 sequence type. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 fIR16 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including the upstream fIR16 sequence type. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 fIR20 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including the upstream fIR20 sequence type. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
$pComP_{IND}$ CmR	Plasmid for allelic replacement at a chromosomal location between ORFs NMB1428 and NMB1429 and inducible expression under the control of the P_{TAC} promoter and the *lacI* repressor. Upstream of the cloning site is a Cm resistance cassette	Ampicillin, Chloramphenicol	Ieva *et al*., J Bacteriol 2005
$pComP_{IND}$ CmR-fHbp var1.1	Plasmid for the complementation of the *fHbp* gene var1.1 in the Com region with an IPTG-inducible P_{TAC}. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	Biagini *et al*., PNAS 2016
$pComP_{IND}$ CmR-fHbp var1.14	Plasmid for the complementation of the *fHbp* gene var1.14 in the Com region with an IPTG-inducible P_{TAC}. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
$pComP_{IND}$ CmR-fHbp var2.16	Plasmid for the complementation of the *fHbp* gene var2.16 in the Com region with an IPTG-inducible P_{TAC}. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
$pComP_{IND}$ CmR-fHbp var2.21	Plasmid for the complementation of the *fHbp* gene var2.21 in the Com region with an IPTG-inducible P_{TAC}. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
$pComP_{IND}$ CmR-fHbp var2.25	Plasmid for the complementation of the *fHbp* gene var2.25 in the Com region with an IPTG-inducible P_{TAC}. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
$pComP_{IND}$ CmR-fHbp var3.28	Plasmid for the complementation of the *fHbp* gene var3.28 in the Com region with an IPTG-inducible P_{TAC}. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
$pComP_{IND}$ CmR-fHbp var3.45	Plasmid for the complementation of the *fHbp* gene var3.45 in the Com region with an IPTG-inducible P_{TAC}. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
$pComP_{IND}$ CmR-fHbp var3.47	Plasmid for the complementation of the *fHbp* gene var3.47 in the Com region with an IPTG-inducible P_{TAC}. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study

Table 6. (continued) Plasmids used in this study**.**

Name	Description	Antibiotic resistance	Reference
pBS-c741 PfHbp -10 box (TACCGC) CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including its upstream promoter region (flR7) mutated in the -10 box: TACCGC. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 PfHbp term -27 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including its upstream promoter region (flR7) mutated in the terminator: strong. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 PfHbp term -27, -10 box (TACCGC) CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including its upstream promoter region (flR7) mutated in the terminator: strong; and in the -10 box: TACCGC. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 PfHbp term -27, spacer1 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including its upstream promoter region (flR7) mutated in the terminator: strong; spacer: CAGTATGCAAAAAAAGA. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 PfHbp term -27, spacer2 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including its upstream promoter region (flR7) mutated in the terminator: strong; spacer: CAATATGCAAAAAAAGA. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 PfHbp term -27, spacer3 CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including its upstream promoter region (flR7) mutated in the terminator: strong; spacer: CAGCATGCAAAAAAAGA. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study
pBS-c741 PfHbp term -27, -35 box (TTGACG) CmR	Plasmid for the *in locus* complementation of the *fHbp* gene including its upstream promoter region (flR7) mutated in the terminator: strong; and in the -35 box: TTGACG. Downstream of *fHbp* is cloned a Cm resistance cassette	Ampicillin, Chloramphenicol	This study

Table 7. Oligonucleotides used in this study. [a] underscored letters indicate restriction enzyme sites.

Name	Sequence[a]	Restriction site	Application	Reference
1869-2F	GAAGAAATCGTCGAAGGCATCAAAC		Amplification and sequencing of the intergenic region of *fHbp* (Fw)	Biagini *et al* ., PNAS 2016
fHbp_32	TGTTCGATTTTGCCGTTTCCCTG		Amplification and sequencing of the intergenic region of *fHbp* (Rev)	Masignani *et al* ., J Exp Med 2003
fHbp_A1	GACCTGCCTCATTGATG		Sequencing of the intergenic region of *fHbp* (Fw)	Masignani *et al* ., J Exp Med 2003
IS-seq-fHbp-F1	GAACTCTTTGCCGTTATC		Sequencing of the intergenic region of *fHbp* when IS30 insertion element was present	This study
IS-seq-fHbp-F2	CTTGGCGAAGGTAGCG		Sequencing of the intergenic region of *fHbp* when IS30 insertion element was present	This study
IS-seq-fHbp-F3	CGTTCGCCTTGGGTC		Sequencing of the intergenic region of *fHbp* when IS30 insertion element was present	This study
IS-seq-fHbp-R1	GCCGCAAACTCAGTC		Sequencing of the intergenic region of *fHbp* when IS30 insertion element was present	This study
SNP3PfHbp-F	GCATACGCCATATCGGCCTTGCCGCCAAGC		remove a SNP in the 3' of the CDS of fHbp (Fw)	This study
SNP3PfHbp-R	GCTTGGCGGCAAGGCCGATATGGCGTATGC		remove a SNP in the 3' of the CDS of fHbp (Rev)	This study
TACCAC_TACCAT-F	GCGGTATGCAAAAAAAGATACCATAACCAAAATGTTTATATATTATC		mutate box -10 TACCAT (Fw)	This study
TACCAC_TACCAT-R	GATAATATATAAACATTTTGGTTATGGTATCTTTTTTTGCATACCGC		mutate box -10 TACCAT (Rev)	This study
TACCAC_TACCGC-F	GCGGTATGCAAAAAAAGATACCGCAACCAAAATGTTTATATATTATC		mutate box -10 TACCGC (Fw)	This study
TACCAC_TACCGC-R	GATAATATATAAACATTTTGGTTGCGGTATCTTTTTTTGCATACCGC		mutate box -10 TACCGC (Rev)	This study
GACGACA_GACGGCA-F	GAACCGCCGTTCGGACGGCATTTGATTTTTGCTTC		mutate terminator from weak to strong (Fw)	This study
GACGACA_GACGGCA-R	GAAGCAAAAATCAAATGCCGTCCGAACGGCGGTTC		mutate terminator from weak to strong (Rev)	This study
CGGTATG_CAGTATG-F	CCTGCCTCATTGATGCAGTATGCAAAAAAAGATACC		mutate spacer into spacer 1 (Fw)	This study
CGGTATG_CAGTATG-R	GGTATCTTTTTTTGCATACTGCATCAATGAGGCAGG		mutate spacer into spacer 1 (Rev)	This study
CAGTATG_CAATATG-F	CCTGCCTCATTGATGCAATATGCAAAAAAAGATACC		mutate spacer into spacer 2 (Fw)	This study
CAGTATG_CAATATG-R	GGTATCTTTTTTTGCATATTGCATCAATGAGGCAGG		mutate spacer into spacer 2 (Rev)	This study
CAGTATG_CAGCATG-F	CCTGCCTCATTGATGCAGCATGCAAAAAAAGATACC		mutate spacer into spacer 3 (Fw)	This study
CAGTATG_CAGCATG-R	GGTATCTTTTTTTGCATGCTGCATCAATGAGGCAGG		mutate spacer into spacer 3 (Rev)	This study
TTGATG_TTGACG-F	CTTTGACCTGCCTCATTGACGCGGTATGCAAAAAAAG		mutate box -35 TTGACG (Fw)	This study
TTGATG_TTGACG-R	CTTTTTTTGCATACCGCGTCAATGAGGCAGGTCAAAG		mutate box -35 TTGACG (Rev)	This study
iPCRprom-F	caagggcgaattgaaccaaatCGTCAAATAACAGGTTG		iPCR - universal for the 11 most represented promoter types (including leader peptide) (Fw)	This study
iPCRprom-R	CCCCCTCCGCTGCTGCAGGCGGTCAGAATCAG		iPCR - universal for the 11 most represented promoter types (including leader peptide) (Rev)	This study
vPCRpBSc741-F	CAGCAGCGGAGGGGGTGGTGTCGCCGCCGAC		vPCR - pBS-c741 wt CmR (Fw)	This study
vPCRpBSc741-R	ATTTGGTTCAATTCGCCCTTGgcataacggcttgcc		vPCR - pBS-c741 wt CmR (Rev)	This study
741-F2	ggattccatatgGTGAATCGAACTGCCTTC	NdeI	Amplification fHbp var1.1 (Fw)	Seib *et al.* , Infect Immun 2011
741-R2	ccaatgcatTTATTGCTTGGCGGCAAG	NsiI	Amplification fHbp var1.1 (Rev)	Seib *et al.* , Infect Immun 2011
EP1For1.1	CGCggatccatatgGTGAATCGAACTGCCTTC	NdeI	Amplification fHbp var1.14 and var3.28 (Fw)	Seib *et al.* , Infect Immun 2011
EP5RV1.4	TGCATGCATTTACTGCTTGGCGGCAAG	NsiI	Amplification fHbp var1.14 (Rev)	Seib *et al.* , Infect Immun 2011
EP2For1.4	CGCggatccatatgGTGAACCGAACTGCCTTC	NdeI	Amplification fHbp var2.16 and var2.25 (Fw)	Seib *et al.* , Infect Immun 2011
EP6RV2.1	TGCATGCATCTACTGTTTGCCGGCGAT	NsiI	Amplification fHbp var2.16, var2.25 and var3.28 (Rev)	Seib *et al.* , Infect Immun 2011
2.21 fw	ATTCGcatatgGTGAACCGAACTGCCTTCTGCTGCC	NdeI	Amplification fHbp var2.21 (Fw)	This study
2.21 rev	ATTCGatgcatCTACTGTTTGCCGGCGATGCCG	NsiI	Amplification fHbp var2.21 (Rev)	This study
3.45 fw	ATTCGcatatgGTGAACCGAACTGCCTTCTGCTG	NdeI	Amplification fHbp var3.45 (Fw)	This study
3.47 fw	ATTCGcatatgGTGAACCGAACTACCTTCTGTTG	NdeI	Amplification fHbp var3.47 (Fw)	This study
3.45/47 rev	ATTCGatgcatCTACTGTTTGCCGGCGATGC	NsiI	Amplification fHbp var3.45 and var3.47 (Rev)	This study
CmR-down	GCACTTCTATACTCTCTGTCG		Complementation check *in locus* (Fw), downstream recombination	This study
complcheck-dsGENOME-R	CTGATAATCGCTCAAACG		Complementation check *in locus* (Rev), downstream recombination	This study
COM-C-Fw	CCTCGAGCCGCTGACCGAAGG		Complementation check in the Com region (Fw), upstream recombination	This study
CM-UP-C	GGTCGAAATACTCTTTTCGTGTCC		Complementation check in the Com region (Rev), upstream recombination	This study
COM-C-Rev	ACCGGCATCGGCAACTACAC		Complementation check in the Com region (Rev), downstream recombination	This study
pRTNM_nmb1869U-F	GTATCGACCGCATCAAAG		qRT-PCR *cbbA* (Fw) and complementation check *in locus* (Fw), upstream recombination	This study
pRTNM_nmb1869U-R	TGACTTTCAGCCATTCTTG		qRT-PCR *cbbA* (Rev)	This study
pRTNM_3P-1869-F	CGAATTGAACCAAATCGTC		qRT-PCR intergenic region (Fw)	This study
pRTNM_IG-R	CAATGAGGCAGGTCAAAG		qRT-PCR intergenic region (Rev)	This study
pRTfHbpU.F	GGCTTGCCGATGCACTAAC		qRT-PCR *fHbp* (Fw) and complementation check in the Com region (Fw), downstream recombination	This study
pRTfHbpU.R	GTTTTTTCCGCACCTTGTGC		qRT-PCR *fHbp* (Rev) and complementation check *in locus* (Rev), upstream recombination	This study
pRTNM16sII.F1	GTGGGGAATTTTGGACAATG		qRT-PCR *16S RNA* (Fw)	This study
pRTNM16sII.R1	CAACAGCCTTTTCTTCCCTG		qRT-PCR *16S RNA* (Rev)	This study

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