Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

BIOLOGIA CELLULARE E MOLECOLARE

Ciclo XXIX

Settore Concorsuale SSC 05/E2

Settore Disciplinare SSD BIO/11

Investigating the molecular mechanisms of *Neisseria meningitidis* antigen regulation: determining a switch between colonization and invasion

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Esame finale anno 2017

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1. Abstract

Neisseria meningitidis colonizes the nasopharynx of humans and pathogenic strains can disseminate into the bloodstream causing septicemia and meningitis. Neisserial Heparin Binding Antigen (NHBA) and Neisserial Adhesin A (NadA) are part of a multicomponent vaccine against *N. meningitidis* serogroup B, called 4CMenB (Bexsero™).

NHBA is a surface-exposed lipoprotein which is expressed by all *N. meningitidis* strains in different isoforms. NHBA harbors an arginine-rich motif through which it is able to bind heparin-like molecules, increasing adherence to host tissues and heparin-mediated serum resistance. We determined that temperature controlled the expression of NHBA in all strains tested, regardless of the clonal complex or peptide isoform expressed. NHBA expression was significantly increased at 30-32°C compared to 37°C, the temperature standardly used for *in vitro* culturing. An increase in NHBA expression at lower temperatures was measurable both at protein and RNA levels and was also reflected by a higher surface exposure of this antigen. A detailed molecular analysis indicated that multiple molecular mechanisms are responsible for the thermoregulated NHBA expression. The comparison of RNA steady state levels in cells cultured at 30°C and 37°C demonstrated an increased RNA stability/translatability at lower temperatures. Furthermore, protein stability was also impacted resulting in higher NHBA stability at lower temperatures. Increased NHBA expression resulted in more efficient killing as shown by serum bactericidal assay (SBA). Mimicking the invasive condition, we investigated the NHBA expression in response to the presence of serum. We showed that the presence of human serum has contrasting effects on NHBA expression, resulting in transient up-regulation of NHBA at transcriptional level, however the protein is rapidly processed likely by complement proteases. We propose a model in which NHBA regulation in response to temperature downshift might reflect the bacterial adaptation during the initial step of host-bacterial interaction and might also explain higher susceptibility to anti-NHBA antibodies in the nasopharynx niche. On the other hand, the initial up-regulation and the high processing of NHBA might play a role during the first steps of invasive disease. All together these data describe the importance of NHBA both as virulence factor and as vaccine antigen during neisserial colonization and invasion.

In the second part of the thesis, we compared genetically engineered outer membrane vesicles and recombinant proteins, as delivery systems of protective antigen. Using NadA as model antigen, we determined that OMV overexpressing NadA produced by homologous (MenB) or heterologous (*E.coli*) bacterial strains, are able to elicit a higher functional antibody response respect to the recombinant protein *per se*, despite the comparable anti-NadA titres elicited. The differences in functionality might be due to different IgG subclasses distribution. Moreover, OMV overexpressing NadA are able to elicit antibodies that inhibit NadA-mediated adhesion on the host cells surface, in a much more efficient way respect to the recombinant protein formulation.

These results indicate that the antigen delivered on OMV triggers a good functional immune response. This preliminary characterization supports the use of OMV as delivery systems for next generation vaccine design and remark the great potential of NadA as model antigen.

2. Introduction

2.1. Meningococcal disease

Invasive meningococcal disease is characterized by a rapid onset and progression to meningitis and/or sepsis which can lead to death within hours. The etiological agent of this devastating disease is *Neisseria meningitidis*, otherwise known as meningococcus. The first report of meningococcal disease is dated back to 1887 by Anton Weichselbaum, who described the meningococcal infection of the cerebrospinal fluid of a patient (Weichselbaum, A. 1887). Each year there are an estimated 1.2 million cases of invasive meningococcal disease and 135,000 deaths (WHO 2010). Despite the availability of antibiotic treatment, approximately 10 to 14% of people who contract meningococcal disease die, and the rate increases to 40-55% in the case of sepsis (Brandtzaeg, P. et al. 2005, Rosenstein, N.E. et al. 2001). Furthermore, approximately 11 to 19% of individuals surviving the disease often suffer from permanent sequelae, including neuro-developmental deficits, hearing loss, ataxia, hemiplegia, seizures and limbs loss (Kaplan, S.L. et al. 2006, Rosenstein, N.E. et al. 2001, Thompson, M.J. et al. 2006, WHO 2010).

Multiple studies have demonstrated that carriage rates are very low in the first few years of life, but rise during adolescence, reaching peaks of 10-35%, and decreasing to less than 10% in older groups (Caugant, D.A. et al. 2007, Claus, H. et al. 2005). In contrast to 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 persons during epidemics (Caugant, D.A. et al. 2009, Stephens, D.S. et al. 2007). Most cases of meningococcal disease occur in otherwise healthy individuals without identified risk factors and what determines the transition from colonization to invasive disease is not yet fully understood. However, certain biological, environmental and social factors have been associated with an increased risk of disease. 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 (Cohn, A.C. et al. 2010). Several studies demonstrated that both host and pathogen factors influence the development of the disease, such as human genetic polymorphisms, impaired immune system, microbial virulence factors, as well as environmental conditions facilitating exposure and

acquisition, and naso- and oro-pharyngeal irritation caused by smoking and respiratory tract infection (Brigham, K.S. et al. 2009, Davila, S. et al. 2010, Goldschneider, I. et al. 1969, Harrison, L.H. 2006, Imrey, P.B. et al. 1995, Rosenstein, N.E. et al. 2001, Zuschneid, I. et al. 2008). The unspecific symptoms at the onset and the early stage of infection, like headache, fever and rash, can implicate an arduous diagnosis. Due to the rapid progression of this life-threatening pathology, vaccination represents the unique effective public health response.

2.2. *Neisseria meningitidis*: pathogen and pathogenesis

N. meningitidis is a strictly human, Gram-negative β-proteobacterium member of the Neisseriaceae family. It is an aerobic, non-motile and non-sporulating diplococcus (Figure 2.1), usually encapsulated and piliated.

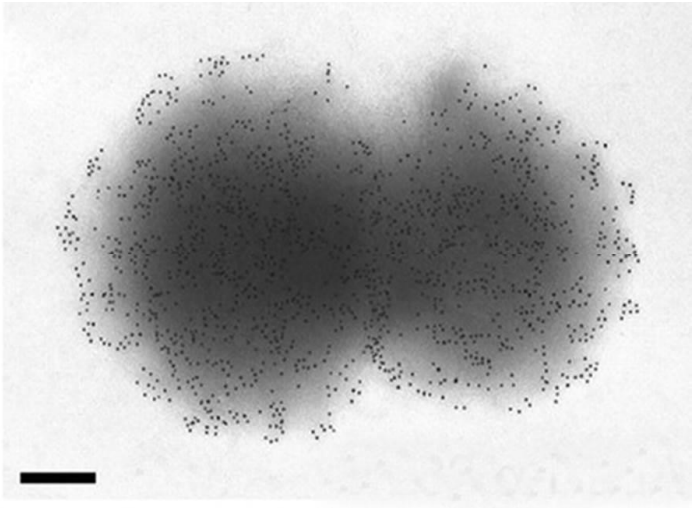


Figure 2.1. Immuno-gold labelling and transmission electron microscopy of *Neisseria meningitidis*. Analysis of the strain was performed with antisera raised against the NadA adhesin. Scale bars: 200 nm (from (Pizza, M. et al. 2000)

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 (Johswich, K.O. et al. 2012). On the basis of the bacterial polysaccharide capsule, *N. meningitidis* can be classified into at least thirteen serogroups: A, B, C, E-29, H, I, K, L, W, X, Y, Z and 29E (Branham, S.E. 1953). Among them, six serogroups (A, B, C, X, Y and W) are responsible for more than 90% of meningococcal disease worldwide and are thus considered pathogenic (Boisier, P. et al. 2007, Frasch, C.E. 1989, Jarvis, G.A. et al. 1987, Stephens, D.S. et al. 2007). Meningococci are further classified into serotypes and serosubtypes 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 (Maiden, M.C. et al. 1998). 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 an identification number is assigned. 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) (Urwin, R. et al. 2003). 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 (Caugant, D.A. 2008, Maiden, M.C. 2008). However, the reasons of this enhanced pathogenic phenotype are yet unknown.

The pathogenesis of *N. meningitidis* is a complex multi-stage process (Figure 2.2).

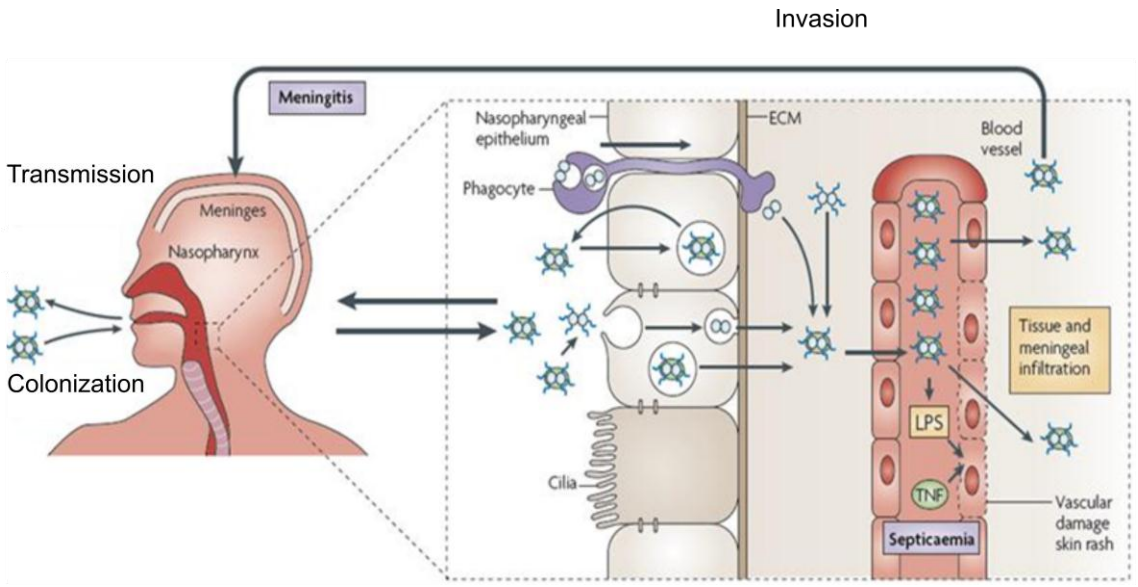


Figure 2.2 Stages in the pathogenesis of *Neisseria meningitidis*. *N. meningitidis* may be acquired through the inhalation of respiratory droplets. The organism establishes intimate contact with non-ciliated mucosal epithelial cells of the upper respiratory tract, where it may enter the cells briefly before migrating back to the apical surfaces of the cells for transmission to a new host. Besides transcytosis, *N. meningitidis* can cross the epithelium either directly following damage to the monolayer integrity or through phagocytes in a 'Trojan horse' manner. In susceptible individuals, once inside the blood, *N. meningitidis* may survive, multiply rapidly and disseminate throughout the body and the brain. Meningococcal passage across the brain vascular endothelium (or the epithelium of the choroid plexus) may then occur, resulting in infection of the meninges and the cerebrospinal fluid (See text for details) (Virji, M. 2009).

The human nasopharynx is the natural biological niche colonized by *N. meningitidis* and transmission to new hosts is facilitated through aerosol droplets (Caugant, D.A. and Maiden, M.C. 2009), as well as direct contact. Acquisition is generally asymptomatic, but infrequently may result in local inflammation, invasion of mucosal surfaces, access to the bloodstream and fulminant sepsis or focal infections such as meningitis (Stephens, D.S. et al.). Meningococcal disease usually occurs 1–14 days after acquisition of the pathogen (Rosenstein, N.E. et al. 2001), after which the carrier state may be established for a period that vary between days to months. From an evolutionary perspective, the interactions of meningococci and the human nasopharynx are key events. Meningococcal carriage and transmission, not disease,

determine the global variation and composition of the natural population of meningococci.

Colonization is an essential but considerably challenging process in meningococcal survival, and therefore a prerequisite for strain carriage as well as for establishing invasive disease (Stephens, D.S.).

Initially, *N. meningitidis* preferentially adheres to relatively non-ciliated or damaged areas of the epithelial barrier. Pili and outer membrane opacity proteins such as Opa and Opc play a major role for meningococcal adhesion to human cells (Hill, D.J. et al. 2012, Kallstrom, H. et al. 2001). Upon contact with human cells, the meningococcus forms microcolonies and adheres using filamentous structures named type IV pili (T4P) which may recognize the host receptor CD46 (Kallstrom, H. et al. 2001), forming a layer tightly attached to host cells (Nassif, X. et al. 1997). After this step, the capsule, which masks the OM proteins via steric hindrance, is lost or down-regulated due to cell-contact induced repression (Deghmane, A.E. et al. 2002) or selection of low or no-capsule expressing bacteria caused by phase variation (Hammerschmidt, S. et al. 1996). The absence of the capsule reveals a variety of redundant adhesins, which mediate a close adherence of the bacteria to host epithelial cells (Stephens, D.S. 2009). In fact, other minor adhesins such as *Neisseria* adhesin A (NadA) (Capecchi, B. et al. 2005), *Neisseria* hia/hsf homologue (NhhA) (Scarselli, M. et al. 2006), Adhesin complex protein (Acp) (Hung, M.C. et al. 2013), Adhesion and penetration protein (App) (Serruto, D. et al. 2003), Meningococcal serine protease A (MspA) (Turner, D.P. et al. 2006) and *Neisserial* heparin binding antingen (NHBA) (Vacca, I. et al. 2016) have been shown to significantly contribute towards *N. meningitidis* colonization of the human nasopharynx.

The interaction of bacterial opacity proteins, Opa and Opc, with CD66/CEACAMs and integrins respectively, on the surface of epithelial cells triggers meningococcal internalization (Gray-Owen, S.D. et al. 2006). Meningococci are capable of intracellular replication and this is in part due to 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) (Perkins-Balding, D. et al. 2004). This intracellular lifestyle gives the bacteria the opportunity to evade the host immune response as well as to find new source of nutrients. Occasionally, bacteria can cross the mucosal epithelial barrier of susceptible individuals, either through

transcytosis or through phagocytes in a "Trojan horse" manner, or directly following damage to the monolayer integrity (Virji, M. 2009), and eventually enter the bloodstream. In healthy individuals, bacteria that cross the mucosal epithelium are eliminated by serum bactericidal activity. Nonetheless, survival within human blood relies upon different mechanisms and is dependent on their capability to evade the immune response and to acquire nutrients. Indeed, the up-regulation of capsule expression prevents antibodies and complement deposition (Uria, M.J. et al. 2008) 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) (Madico, G. et al. 2006), or by the Neisserial surface protein A (NspA) (Lewis, L.A. et al. 2010), and by the Porin B (PorB) (Lewis, L.A. et al. 2013), or the recruitment of complement regulators, such as the C4-binding protein, which is bound by Porin A (PorA) (Jarva, H. et al. 2005). Once inside the bloodstream, meningococci can multiply slowly and eventually cross the blood-brain barrier, causing the infection of meninges and cerebrospinal fluid (Nassif, X. 2009). Otherwise, in case of rapid multiplication within the blood, the bacteria cause septicemia or meningococcemia (Rosenstein, N.E. et al. 2001, Tinsley, C. et al. 2001).

Overall, the onset of meningococcal disease can be seen as a failed relationship between the meningococcus and the host. While factors that trigger meningococcal entrance in the bloodstream are not yet fully understood, they are likely dependent on both the host and pathogen sides and include impairing of the integrity of the human nasopharyngeal mucosa, the lack of a protective immune response and microbial factors influencing virulence (Caugant, D.A. and Maiden, M.C. 2009, Stephens, D.S. et al. 2007).

2.3. Meningococcal virulence factors

Within the host *N. meningitidis* colonizes and invades diverse sites which represent different niches with respect to nutrients, environmental factors and competing microorganisms. The pathogen is subjected to constant selective pressures and its ability to rapidly adapt its metabolism and cellular composition to environmental changes is essential for its survival (Hill, D.J. et al. 2010). Bacteria achieve adaptation to the environment either by changing their genotype (genome plasticity) or by transient

alterations in gene expression. These two mechanisms are complementary and both lead to phenotypic variations.

The genome variability is also assured by the horizontal gene transfer that occurs with a relatively high frequency considering the high natural competence of meningococci. Instead, the genome plasticity is guaranteed by the abundance of mobile elements that represent the 10% of the entire genome (Parkhill, J. et al. 2000). Furthermore, other interesting phenomenon that significantly contributes to meningococcal genome plasticity is phase variation. It represents an adaptive process by which *N. meningitidis* undergoes stochastic, frequent and reversible phenotypic changes as consequence of genetic alterations in specific loci, altering mainly virulence-associated, surface-exposed antigens such as outer-membrane proteins PorA, Opc, Opa, pili and specific adhesins, as well as LOS and capsule (Davidsen, T. et al. 2006, Feil, E.J. et al. 2001, Metruccio, M.M. et al. 2009, Moxon, E.R. et al. 1998). Meningococcal strains associated with disease have high frequency of phase variation, indicating that varying surface-exposed components provides substantial benefits during transmission between hosts (Richardson, A.R. et al. 2002).

Distinct from phase variation, antigenic variation is a mechanism of immune evasion where bacteria express different moieties of functionally conserved molecules that are antigenically distinct within a clonal population. This process is distinct from phase variation, as only one variant is expressed at any given time, although the cell still contains the genetic information to produce a whole range of antigenic variants. In the pathogenic *Neisseria* species, antigenic variation occurs in several surface components, including type IV pili, LOS and Opa proteins (Davidsen, T. and Tonjum, T. 2006).

The virulence of *N. meningitidis* is influenced by multiple factors that are mainly located in the outer membrane (Figure 2.3).

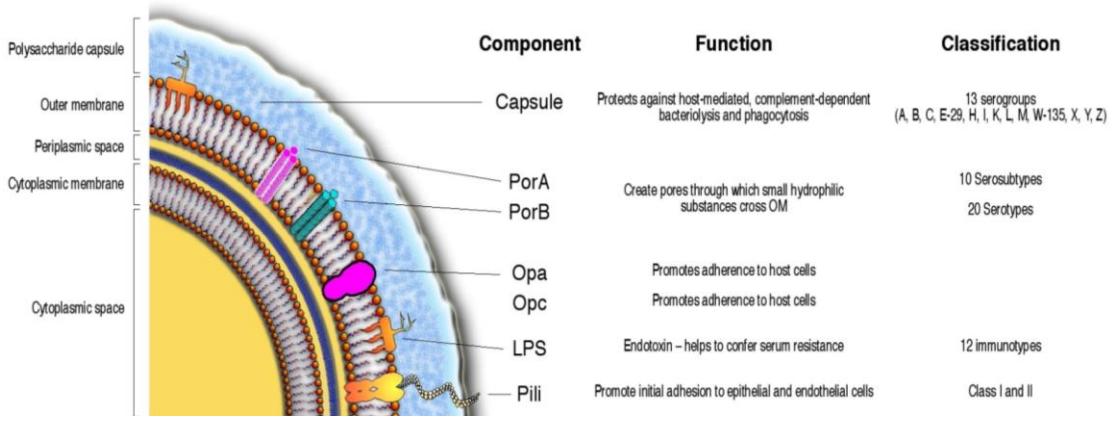


Figure 1.3 Meningococcal cell compartments. Schematic representation of the different bacterial compartments and of the main components of the outer membrane, together with their known function (adapted from Rosenstein NE, 2001).

The main virulence factor is the polysaccharidic capsule, which represents a barrier that protects the bacterium from the host innate and adaptive immune system (Schneider, Exley, Ram, Sim, & Tang, 2007; Vogel & Frosch, 1999). It also defends meningococcus from desiccation during airborne transmission between hosts (Virji, M. 2009); (Romero, J.D. et al. 1997). Its expression is phase variable (Hammerschmidt, S. et al. 1996) 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 (Swartley, J.S. et al. 1997). In meningococcus, LPS are referred to as lipooligosaccharide (LOS) because of the presence of repeating short saccharides instead of long-chain saccharides. LOS is the major constituent of the outer leaflet of the meningococcal outer membrane (OM), responsible for the physical integrity and proper functioning of the membrane and required for resistance of *N. meningitidis* to complement (Geoffroy, M.C. et al. 2003). LOS comprises an inner and outer oligosaccharide core attached to the lipid A portion that anchors the LOS in the outer leaflet of the OM. Lipid A is responsible for the toxicity of LOS due to its ability to bind to different Toll-like receptors on monocytes and on dendritic cells triggering the secretion of various inflammation mediators (Brandtzaeg, P. et al. 2001); (Braun, J.M. et al. 2002). 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 (Jennings, M.P. et al. 1999). 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 (Pinner, R.W. et al. 1991, Virji, M. et al. 1992). Pili represent the major contributor to the adhesive property of the capsule (Stephens, D.S. et al. 1981, Virji, M. et al. 1991) and in addition they are involved in the uptake of foreign DNA from the extracellular environment, hence increasing transformation frequency and consequently genetic adaptability (Helaine, S. et al. 2007).

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 (Virji, M. et al. 1992). 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 (Virji, M. 2009). The Neisserial adhesin A (NadA) is a surface-exposed member of the Oligomeric coiled-coil adhesin family of bacterial Trimeric Autotransporter adhesins (El Tahir, Y. et al. 2001, Helaine, S. et al. 2007). NadA mediates adhesion to and invasion of human epithelial cells (Capecchi, B. et al. 2005), 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. NHBA has been recently shown to participate during the colonization process by increasing adherence to host tissues by binding glycosaminoglycans (Vacca, I. et al. 2016), and mediating biofilm formation ((Arenas, J. et al. 2013)). The Meningococcal surface fibril (Msf), previously termed Neisseria hia/hsf homologue A (NhhA) (Peak, I.R. et al. 2000, Weynants, V.E. et al. 2007), mediates adhesion to epithelial cells and to components of the extracellular matrix, even though at low levels (Scarselli, M. et al. 2006). 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 (Griffiths, N.J. et al. 2011), and its role in inhibiting phagocytosis, inducing macrophages apoptosis and protecting bacteria against complement-mediated killing has been suggested (Sjolinder, H. et al. 2008, Sjolinder, M. et al. 2012). Two homologous autotransporters,

the Adhesion penetration protein (App) and the Meningococcal serine protease A (MspA) are involved in the bacterial interaction to epithelial cells (Serruto, D. et al. 2003, Turner, D.P. et al. 2006) and also in the apoptosis of dendritic cells (Khairalla, A.S. et al. 2015). Glycolipid adhesins such as members of the Multiple adhesin family (Maf) may contribute to the bacterial interaction with host cells (van Putten, J.P. et al. 1998). Interestingly they are found to be associated with genomic islands present only in pathogenic Neisseria species, both meningococcus and gonococcus (Jamet, A. et al. 2015).

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 (Orihuela, C.J. et al. 2009); moreover, PorA elicits a protective immune response in humans (Holst, J. et al. 2009, Wedege, E. et al. 1998), while PorB might be involved in the immune system evasion by binding the human fH (hfH) (Lewis, L.A. et al. 2013). The regions of PorA that generate the immune response are loops 1 and 4, named VR1 and VR2, that are hyper variable among strains. OMV based vaccines, such as 4CMenB, use PorA as significant antigen generating bactericidal immune responses. However, due to the hyper variability of the immune-dominant regions, PorA-based vaccines provide protection only against strains expressing homologous PorA serosubtypes (see below).

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 (Figueroa, J. et al. 1993). 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 have shown to bind the fH, fHbp (Madico, G. et al. 2006), NspA (Lewis, L.A. et al. 2010) and PorB (Lewis, L.A. et al. 2013). Strains lacking both fHbp and NspA were not able to bind fH and indeed were more susceptible to complement-dependent killing (Echenique-Rivera, H. et al. 2011, Lewis,

L.A. et al. 2010). In addition, the observed binding of heparin from the NHBA may increase bacterial serum resistance due to the potential interactions of heparin with fH (Serruto, D. et al. 2010).

2.4. Anti-meningococcal vaccines

Due to its rapid progression and the difficulties to diagnose it (Rosenstein, N.E. et al. 2001, Thompson, M.J. et al. 2006), 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*. Different meningococcal vaccines have been developed against the distinct serogroups (Zahlanie, Y.C. et al. 2014). There are a number of polysaccharide and conjugate meningococcal vaccines in use against serogroups A, C, Y and W135. The tetravalent vaccine composed of purified capsular polysaccharides, although efficacious in adolescent and adults, is poorly immunogenic in infants and fails to induce immunological memory. However, when conjugated to a carrier protein, capsule polysaccharides show a greatly improved immunogenicity in young infants (Granoff, D.M. et al. 2007, Nassif, X. 2009, Virji, M. 2009). Monovalent, bivalent, and tetravalent polysaccharide conjugative vaccines are available and effective against meningococcal serogroups A, C, Y and W-135 (Zahlanie, Y.C. et al. 2014); http://www.who.int/ith/vaccines/meningococcal/en/]. The first trials conducted in the United Kingdom with the meningococcus C conjugate showed a dramatic decline in the incidence of serogroup C disease in all age groups that received the vaccine (Borrow, R. et al. 2000, Miller, E. et al. 2001) with an efficacy of 97 and 92 per cent for teenagers and toddlers, respectively (Ramsay, M.E. et al. 2001).

In contrast, the group B capsule polysaccharide is not suitable as vaccine antigen. It consists of a homolinear polymer of α(2→8)N-acetyl neuraminic acid, also known as polysialic 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 (Finne, J. et al. 1987, Finne, J. et al. 1983). Therefore, efforts to develop a vaccine against meningococcus serogroup B (MenB) focused mainly on non-capsular antigens, such as proteins or LOS. The principal challenge has been to identify surface-exposed non-capsular antigens that are safe, antigenically conserved and that elicit a broad Serum Bactericidal Antibody (SBA) response. Licensed and promising group B vaccine approaches are discussed below.

2.4.1. Licensed vaccines against MenB

Detergent-extracted OMV vaccines (dOMV)

In order to control outbreaks caused by specific MenB strains vaccines composed of dOMV have been successfully employed in Norway (Fredriksen, J.H. et al. 1991), Cuba (Sierra, G.V. et al. 1991), Chile (Boslego, J. et al. 1995) and New Zealand (Oster, P. et al. 2005). The detergent treatment removes the toxic LOS, but it also extracts other desirable antigens such as lipoproteins. Consequently, the porin protein PorA results to be the immuno-dominant antigen (Martin, D.R. et al. 2006, Tappero, J.W. et al. 1999). Despite dOMV vaccines resulted to be safe and to induce good functional responses in humans, the immune response elicited is effective only against strains expressing the same PorA serosubtype, due to PorA antigenic variability (van der Ley, P. et al. 1991). Therefore, dOMV vaccines are well-suited to control local, clonal outbreaks but they do not confer broad protection.

4CMenB

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 antigenically conserved among strains and elicit a bactericidal serum response. This approach led to the development of the four component recombinant protein vaccine 4CMenB (Giuliani, M.M. et al. 2006, Giuliani, M.M. et al. 2010). 4CMenB contains five Genome-derived Neisseria Antigens (GNA) formulated together with the dOMV component from the NZ98/254 strain (Martin, D.R. et al. 2006). Based on their ability to induce broad protection three major antigens have been selected (Giuliani, M.M. et al. 2006): NadA (Capecchi, B. et al. 2005, Comanducci, M. et al. 2002) is present as single polypeptide, while fHbp (Beernink, P.T. et al. 2008, Masignani, V. et al. 2003) and NHBA (Serruto, D. et al. 2010, Welsch, J.A. et al. 2003) are fused to the conserved meningococcal gene products GNA2091 and GNA1030, respectively. The other two antigens, GNA2091 and GNA1030, are well conserved in *N. meningitidis*, but less functionally characterized than the other antigens (Bos, M.P. et al. 2014, Donnarumma, D. et al. 2015, Muzzi, A. et al. 2013). 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 (Giuliani, M.M. et al. 2006). 4CMenB was licensed in Europe in 2013 and in the U.S. in 2015, following its progression through clinical trials that have demonstrated its safety (Esposito, S. et al. 2014, Prymula, R. et al. 2014, Toneatto, D. et al. 2011) and its efficacy in inducing a protective immune response in infants, children, adolescents and adults against the majority of MenB strains (Gossger, N. et al. 2012, Kimura, A. et al. 2011, McQuaid, F. et al. 2014, Read, R.C. et al. 2014, Santolaya, M.E. et al. 2012, Snape, M.D. et al. 2013, Vesikari, T. et al. 2013).

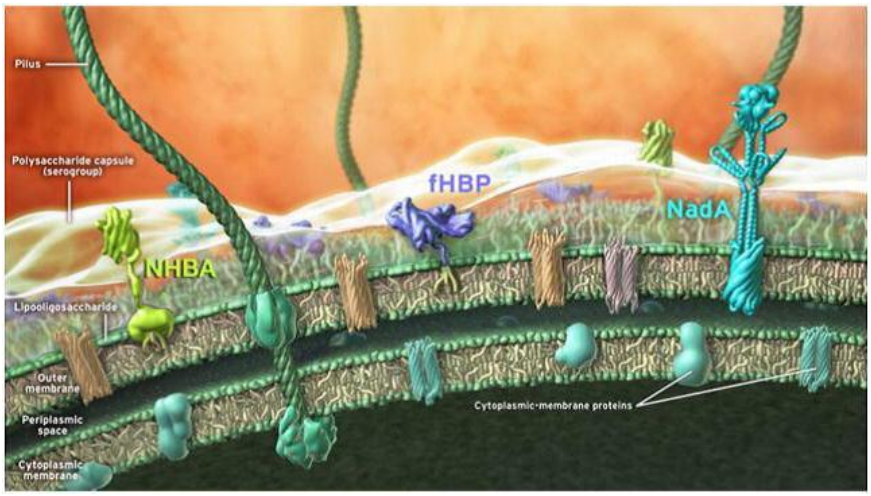


Figure 2.4 Schematic representation of the 4CMenB vaccine antigens on the surface of *N. meningitidis* (from Serruto D, 2012). The different bacterial compartments (outer membrane, periplasmic space, cytoplasmic membrane) and the main antigens identified through reverse vaccinology approach (NHBA, fHbp and NadA) are depicted. Other components of the meningococcal membranes are also shown (pilus, polysaccharide capsule, lipooligosaccharide and integral inner and outer membrane proteins).

Bivalent fHbp-based vaccine or Trumenba

Trumenba was licensed in the U.S. in 2014 for a target population of adolescents and young adults. However, it is not suitable for use in infants considering that it consists of purified lipoproteins known as TLR-2 agonists (Richmond, P.C. et al. 2012). 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 (Fletcher, L.D. et al. 2004).

2.4.2. Investigational MenB vaccines

Recombinant protein vaccines

Several protein antigens have been investigated for their protective ability for use in a MenB vaccine, among which NspA, TbpB, FetA, ZnuD and others (Halperin, S.A. et al. 2007, Hubert, K. et al. 2013, West, D. et al. 2001). The main issue with all of these approaches was the limited cross protective potential of any one antigen. It was clear that a multivalent approach was needed to guarantee a wide protection.

Native Outer Membrane Vesicles (nOMV) vaccines

nOMV are spherical portions of the OM, ~20–250 nm in diameter, produced by Gram negative bacteria. They are spontaneously released during the active growth into the surrounding medium. These portions of the OM bud and detach from the cell, enclosing many native bacterial antigens and periplasmic constituents (Figure 2.5). The vesicles play diverse roles like delivery of virulence factors , modulation of the host immune system during pathogenesis, aid in nutrient acquisition, mediation of cellular communication, surface modifications and the elimination of undesired components that, ultimately, make them a transportable part of the bacterial arsenal and survival system (Collins, B.S. 2011, Kuehn, M.J. et al. 2005, Schwechheimer, C. et al. 2015).

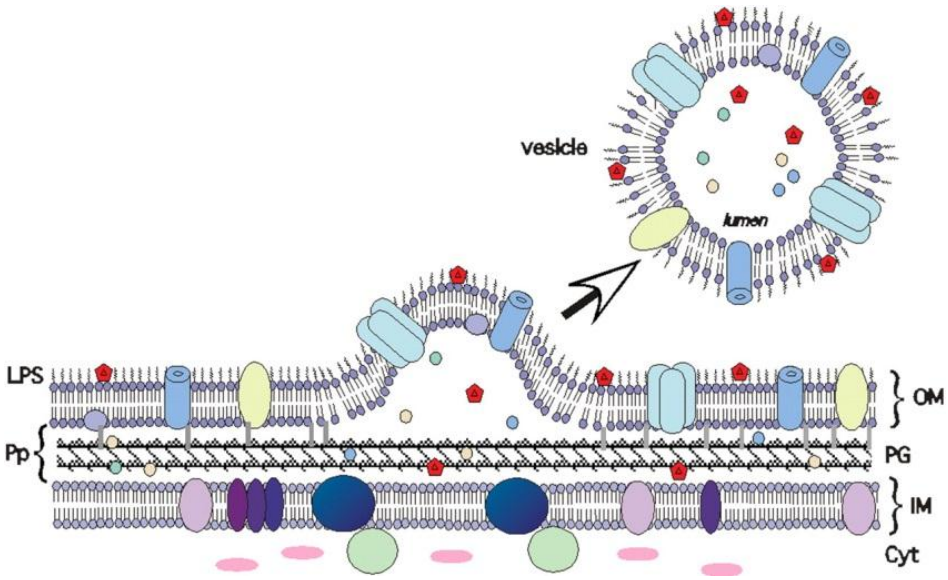


Figure 2.5 Model of native Outer Membrane Vesicles (nOMV) biogenesis. NOMV vesicles are proteoliposomes consisting of OM phospholipids and LPS, a subset of OM proteins and periplasmic (luminal) proteins (Kuehn, M.J. and Kesty, N.C. 2005).

nOMV represent an attractive vaccine platform mimicking the bacterial cell surface. Since nOMV do not undergo to a detergent extraction procedure, like dOMV do, they preserve high amounts of lipooligosaccharide (LOS) as well as protective lipoproteins which would otherwise be removed by the detergent. This was expected to improve immunogenicity and cross-protection provided but it raised safety concerns. Consequently, to prepare safe nOMV vaccines the strain must be genetically engineered to reduce the LOS reactogenicity. The acylation of lipid A molecule is responsible for its endotoxin activity and two mutations (lpxL1 and lpxL2), affecting the reduction of lipid A, have been successfully exploited (Bonvehi, P. et al. 2010, Keiser, P.B. et al. 2011, Keiser, P.B. et al. 2010, Koeberling, O. et al. 2011). The lpxL1 gene, homologous to *E.coli* htrB, encodes for a late acyltransferase of lipid A biosynthesis. Its deletion lead to penta- instead hexa-acetylated molecules, resulting in lower endotoxin activity of LOS (van der Ley, P. et al. 2001). Instead the lpxL2 gene, homologous of *E.coli* lpxLM, encodes for a lauroyl acyltransferase. Its deletion leads to a tetra-acylated lipid A lacking both secondary lauroyl chains.

nOMV vaccines prepared from wild-type strain were poorly immunogenic in mice (Koeberling, O. et al. 2008, Moe, G.R. et al. 2002). Koeberling and colleagues

demonstrated that the level of expression of a key antigen, as fHbp, was a critical parameter to elicit broad serum bactericidal responses (Koeberling, O. et al. 2011). The overexpression of some antigens (Keiser, P.B. et al. 2011) and the removal of the immunodominant PorA antigen (Bonvehi, P. et al. 2010) were two strategies tested in a phase I clinical trial. In the first case, the nOMV vaccine resulted to be safe and immunogenic (Keiser, P.B. et al. 2011); nevertheless, the major contribution to bactericidal activities was from antibodies raised from LOS providing immunotype specific bactericidal responses. This result was suggested to be due to insufficient levels of the antigens over-expressed on the vesicles (Koeberling, O. et al. 2011). In the second case, PorA was deleted to avoid its immune-dominance. The prototype vaccine strain was also engineered to express a truncated form of LOS immunotype L3,7 that is the most common in invasive MenB strain (Scholten, R.J. et al. 1994). This OMV-based vaccine offered good safety but low immunogenicity in healthy young adults (Bonvehi, P. et al. 2010, Weynants, V. et al. 2009).

Chapter 1

NHBA regulation and expression during colonization and invasion

Sensing the environment

Colonization is an essential as well as a considerably challenging process in meningococcal survival, and therefore a prerequisite for strain carriage as well as for establishing invasive disease (Stephens, D.S. 2009). The nasopharyngeal epithelium is a complex ecological niche that poses several hurdles for bacterial colonization and survival. Compounds such as mucus, antimicrobial peptides and immunoglobulins provide physical and biochemical host defenses (Laver, J.R. et al. 2015). Furthermore, this environment is deprived of nutrients such as iron, carbohydrates and oxygen essential for bacterial growth and *N. meningitidis* therefore needs to compete for these limited nutrients with the resident microflora. Taken together, these factors make de novo colonization and survival challenging. *N. meningitidis* is incredibly well adapted to this environment and has developed several mechanisms to control expression of adhesion molecules (Deghmane, A.E. et al. 2002) (Grifantini, R. et al. 2002, Hey, A. et al. 2013), biofilm formation (Arenas, J. et al. 2013), iron acquisition (Larson, J.A. et al. 2002, Schryvers, A.B. et al. 1999), metabolism (Jamet, A. et al. 2009, Mendum, T.A. et al. 2011) (Laver, J.R. et al. 2015), and immune evasion factors (Lomholt, H. et al. 1992, Yazdankhah, S.P. et al. 2004) .

One of the key signals sensed by *N. meningitidis* to determine its environment and to induce the expression of either adhesion or immune evasion factors is temperature (Laver, J.R. et al. 2015). Temperatures within the upper respiratory tract are affected by the passage of air during respiration of the host, the precise anatomical location and the presence of local inflammation (Keck, T. et al. 2000, McFadden, E.R., Jr. et al. 1985) (Figure 2.6). These factors can result in an overall variability of temperature in this niche ranging from 25.3±2.1°C in the nasal vestibule to 33.9±1.5°C in the nasopharynx, generally being several degrees below core body temperature (Keck, T. et al. 2000). *N. meningitidis* has evolved to rapidly and efficiently adapt its metabolism to even minor temperature changes in the environment. During the development of invasive disease, *N. meningitidis* passes from the lower temperatures in the upper airway to the core body temperature of 37°C or higher with a febrile response to infection (Cabanac M. 1990). Within the bloodstream although the increased temperature and the abundance of nutrients promote the fast growth of the bacterium, the presence of the complement cascade components, immunoglobulins and immune cells represent a big threat, meanwhile. Therefore a rapid adaptation to the new environment is required, in fact

approximately 30% of the genes in the genome are dramatically regulated on entry to whole human blood (Echenique-Rivera, H. et al. 2011), triggering an immune evasion response. Key antigens and virulence factors such as capsule biosynthesis (CssA), sialylation of LPS (Lst) and fHbp involved in immune evasion and in establishing invasive disease, show increased expression at 37°C relative to lower temperatures (Loh, E. et al. 2013, Loh, E. et al. 2016). However, the role of lower temperature on the expression of virulence factors has received considerably less attention. Recently, a comparative proteomic study showed that 375 proteins were differentially expressed between 32°C and 37°C (Lappann, M. et al. 2016).

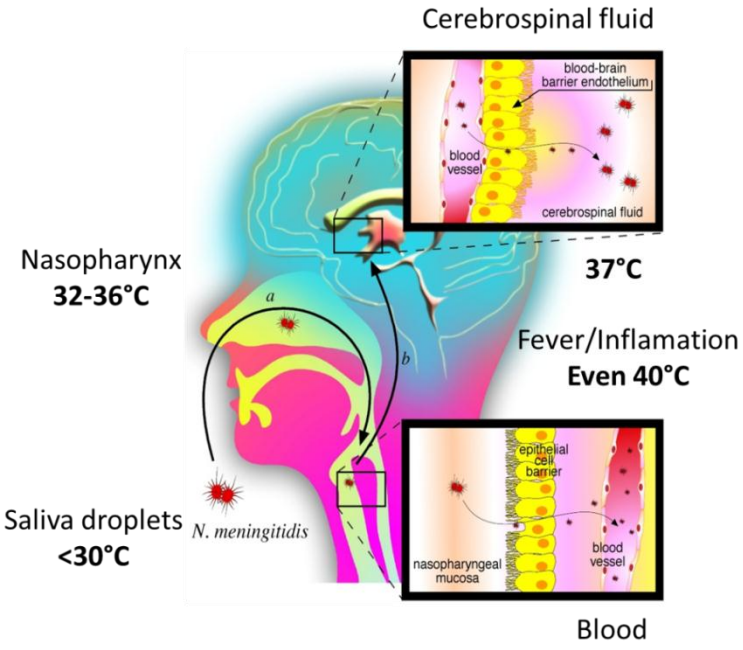


Figure 2.6 Within the host *N. meningitidis* encounters different niches. Temperature is one of the key signal sensed by *N. meningitidis* to determine its environment. The temperatures that *N. meningitidis* encounters during transmission, colonization and invasion are reported.

Neisserial Heparing Binding Antigen (NHBA)

NHBA is a surface exposed lipoprotein that is specific to *Neisseria* species. NHBA is one of the major antigens of the serogroup B meningococcal vaccine, 4CMenB (Serruto, D. et al. 2012), and induces antigen-specific bactericidal antibodies in both animals and humans (Serruto, D. et al. 2010); (Giuliani, M.M. et al. 2010). The *nhba* gene is

ubiquitous in meningococcal strains of all serogroups and it is also found in *N. gonorrhoeae* as well as in different commensal neisserial species (Bambini, S. et al. 2009); (Jacobsson, S. et al. 2006, Muzzi, A. et al. 2013). Analysis of gene sequences from genetically diverse serogroup B strains revealed the existence of more than 400 distinct peptides, which are associated with clonal complexes and sequence types (Comanducci, M. et al. 2002, Jacobsson, S. et al. 2006, Muzzi, A. et al. 2013). Considerable variation is observed at the level of primary amino acid sequence which ranges in length from approximately 430 to 500 residues (Figure 2.7). Most variability is observed at the level of the amino-terminal region, which is annotated as intrinsically unfolded by commonly used structure prediction algorithms (Vacca, I. 2014). In contrast, the carboxyl-terminal region consists of a single 8-stranded anti-parallel beta-barrel structure and is highly conserved (Esposito, V. et al. 2011).

The two domains are linked through an arginine-rich motif which is responsible for NHBA binding to heparin *in vitro* and contributes to increased survival of the un-capsulated *N. meningitidis* in human serum (Esposito, V. et al. 2011, Serruto, D. et al. 2010). Conversely, it has been recently shown that NHBA plays an integral part in binding heparin sulfate proteoglycans on epithelial cells and thus directly mediates adhesion of *N. meningitidis* (Vacca, I. et al. 2016). Furthermore, the presence of this arginine-rich domain was implicated in DNA-binding and could therefore also play a role in the formation of neisserial microcolonies and biofilms (Arenas, J. et al. 2013). NHBA can be processed by the meningococcal protease NalP and human lactoferrin (hLf). Cleavage occurs either upstream and downstream of the NHBA Arg-rich region resulting in one of two possible cleavage fragments termed C2 and C1, respectively (Serruto, D. et al. 2010).

It was also demonstrated that the C-terminal fragment (C2), released upon NalP proteolysis, alters endothelial cell permeability by inducing the internalization of the adherens junction protein VE-cadherin, which is in turn responsible for the endothelial leakage. Thus, the NHBA-derived fragment C2 might contribute to the extensive vascular leakage typically associated with meningococcal sepsis (Casellato, A. et al. 2014).

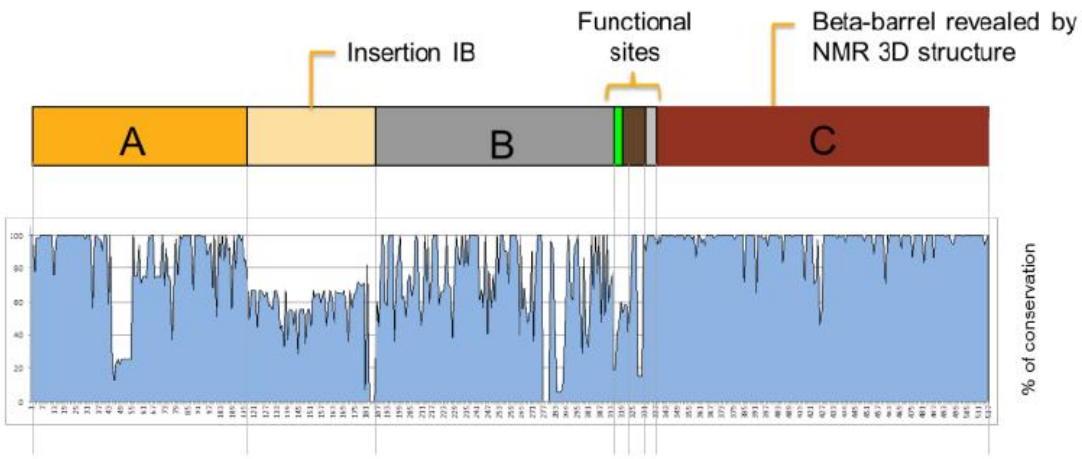


Figure 2.7 NHBA protein schematic view and variability. NHBA protein sequence reflects a modular structural organization, where it is possible to recognize three main domains (A, B and C). The presence of an insertion sequence of 60 amino acids, present only in some of the NHBA peptides (Insertion IB), allows to discriminate between long or short isoforms. Functional sites are represented by the Arg-rich region (in brown), by the NalP cleavage site (in green) and by the human lactoferrin cleavage site (in grey). The C-term of the protein, corresponding to module C is highly conserved and is represented by a beta-barrel structure. The lower graph shows the percentage of amino acid conservation between the different peptides along the protein sequence (adapted from (Vacca, I. 2014)).

The upstream regulatory region of the *nhbA* gene is characterized by the presence of the 150-bp Contact Regulatory Element of Neisseria (CREN) in strains, such as MC58, belonging to clonal complex ST-32. This regulatory element is specific to pathogenic *Neisseria* species and is involved in the induction of the downstream associated genes upon contact with target eukaryotic cells (Deghmane, A.E. et al. 2002). NHBA expression is known to be induced after incubation of bacteria with epithelial cells in the CREN-containing strain MC58, while its expression remains unaltered in the CREN-lacking strain 8013 (Deghmane, A.E. et al. 2003). It was therefore proposed that cell contact increased NHBA levels on meningococcal surface in the ST-32 invasive hypervirulent strains and that increased expression of *nhba* upon host contact might at least partially account for the hypervirulent phenotype of this clonal complex.

3. Results

3.1. NHBA expression and surface exposure are temperature-dependent

NHBA is an important virulence factor for *N. meningitidis* and it is also protective antigen able to elicit an immune response in preclinical and clinical trials (Serruto, D. et al. 2010). Therefore, understanding the mechanisms that drive NHBA regulation is an important goal to better understand *N. meningitidis* pathogenesis and vaccine induced response. We therefore investigated how physiologically relevant temperatures, which mimic the different stages of pathogenesis, may affect the expression levels of NHBA. Strains MC58, M11719 and 8047 were grown overnight on GC agar plates at physiologically relevant temperatures ranging from 28°C up to 40°C. We found that NHBA expression was thermoregulated in an inverse manner to fHbp, with higher expression at lower temperature. Western blot analysis showed that NHBA expression was at its highest level between 28°C and 30°C in all these strains and that its expression decreased markedly with increasing temperatures (Figure 3.1 A). In contrast, fHbp expression was highest at elevated temperatures and decreased with temperature reduction. In order to understand whether temperature regulation of NHBA was conserved among different *N. meningitidis* isolates, we expanded our analysis to a broader panel of strains belonging to different clonal complexes, carrying different variants and also long or short isoforms of NHBA (Figure 3.1 B and Table 3.1). NHBA expression levels were variable among the different strains and showed different processing patterns depending on the strain background and NHBA variant/isoform present (Table 3.1). Despite different variants and expression levels between the strains tested, all strains showed increased levels of NHBA at 30°C compared to 37°C. As NHBA is a surface exposed neisserial protein, we confirmed that increased expression levels of NHBA also resulted in increased levels of NHBA exposed on the bacterial cell surface using flow cytometry (Figure 3.1 C).

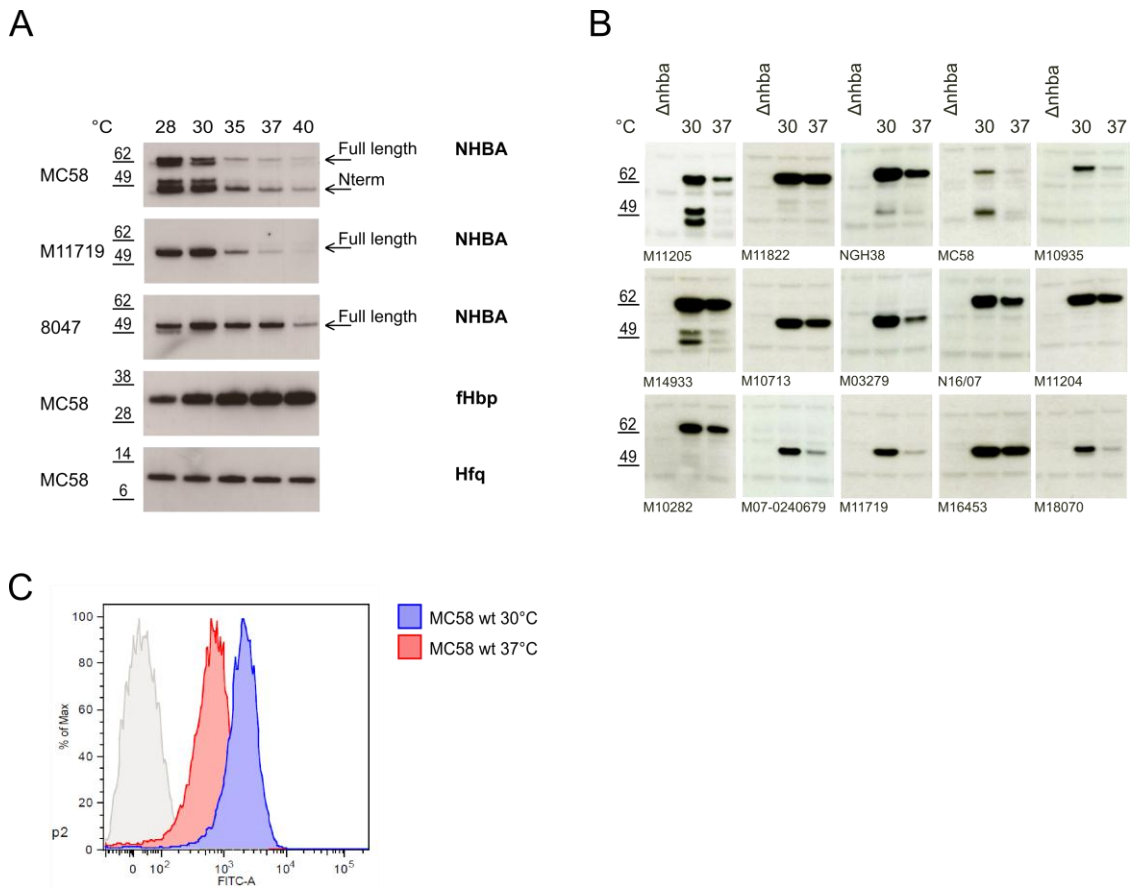


Figure 3.1 NHBA expression and surface exposure are increased at reduced temperatures. (A) The defined strains were grown overnight on GC agar plates at the indicated temperatures. Whole cell lysates were prepared and separated by SDS-PAGE prior to Western Blotting. The indicated proteins were detected using mouse-polyclonal antisera. Hfq served as loading control between different samples. In MC58 strain the full-length protein migrates with an apparent molecular weight of approximately 62 kDa, while other bands at approximately 49 kDa results from the bacterial proteases's processing (Serruto, D. et al. 2010). **(B)** The defined strains were grown in GC broth at 30°C or 37°C until OD_{600} 0.25. Whole cell lysates were prepared and separated by SDS-PAGE prior to Western Blotting. Different strains express different variants and isoforms of NHBA (See table 3.1 for strains details), however the bands specificity is confirmed by the *nhba* deletion mutant (Δnhba) generated for each strain. **(C)** Flow cytometric analysis of strain MC58 showing surface exposure of NHBA at the indicated temperatures.

Strain	Country of origin	Year of isolation	Capsular group	Clonal complex	NHBA peptide variant	NHBA isoform
M11205	USA	2003	B	41/44	p0001	Long
M11822	USA	2004	B	41/44	p0001	Long
NGH38	Norway	1988	B	ua[a]	p0002	Long
MC58	UK	1985	B	32	p0003	Long
M10935	USA	2003	B	35	p0058	Long
M14933	USA	2006	B	32	p0003	Long
M10713	USA	2003	B	41/44	p0010	Short
M03279	USA	1997	B	41/44	p0011	Short
N16/07	Norway	2007	B	41/44	p0029	Long
M11204	USA	2003	B	41/44	p0029	Long
M10282	USA	2003	B	41/44	p0002	Long
M07-0240679	UK	2007	B	269	p0017	Short
M11719	USA	2003	B	162	p0020	Short
M16453	USA	2007	B	41/44	p0144	Short
M18070	USA	2008	B	162	p0020	Short
8047	USA	1978	B	11	p0020	Short

Table 3.1 List of natural strains reported in Figure 3.1. Main characteristics are indicated. For each of them a *nhba* deletion mutant was generated. *Unassigned

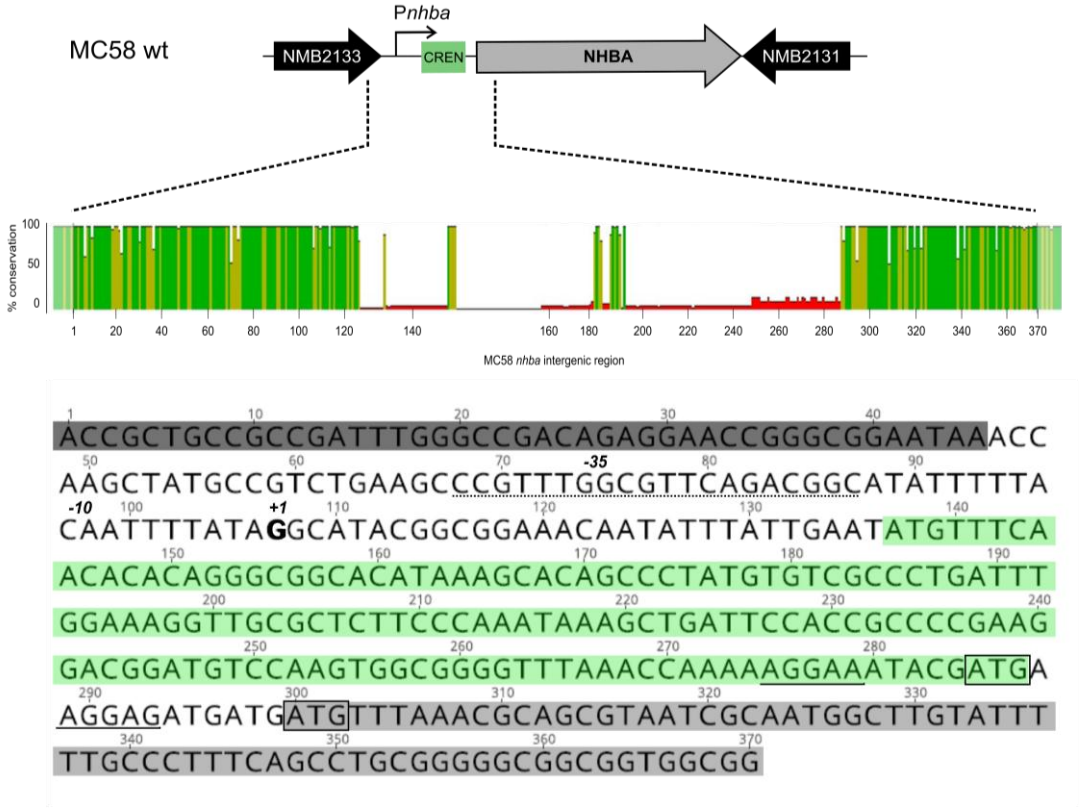
3.2. Mutations and deletions in the 5'UTR and 5'TR of *nhba* affect expression

The *nhba* gene was originally annotated as NMB2132 according to its location within the genome sequence of the strain MC58 (Tettelin, H. et al. 2000). MC58 *nhba* locus schematic view (upper panel) and details of the intergenic region and the 5'TR of *nhba* (lower panel) are reported in Figure 3.2 A. The conservation of this region among all the *Neisseria* species present in the PubMLST database is also reported (mid panel). The region shows a very good conservation among 8373 *Neisseria* strains (green and greeny-brown bars). Red bars indicate the lack of conservation (5.8% strains) which corresponds to the CREN sequence, specific for ST-32.

Upstream and in the same orientation of *nhba* is located NMB2133. A Rho-independent terminator is predicted around 22 nucleotides downstream (Figure 3.2 A lower panel). qRTPCR experiments confirmed that no co-transcription is detectable among NMB2133 and NMB2132 (data not shown). A putative promoter (P*nhba*) was identified upstream the CREN sequence.

The annotated translational start site is boxed in black and the ribosomal binding site is also indicated and underlined. However, in frame with the annotated one and just next to it, two more putative translational start sites were identified (boxed with dashed lines). Moreover, within the CREN sequence and still in frame with the annotated one, another putative translational start site (boxed in green) was identified, carrying also an alternative ribosomal binding site (underlined in green) (Deghmane, A.E. et al. 2003). In order to investigate which one corresponds to the initiation of translation in correlation with temperature changes, a series of site-directed mutagenesis were performed (Figure 3.2 B). As shown by Western blot analysis, small deletions or single base mutations affecting these sites (Mut_1-4) led to decreased or abolished NHBA expression, without affecting thermoregulation (Figure 3.2 C left panel). We identified a T-rich region in the 5'TR of *nhba,* 20 nucleotides downstream to the putative translational start site. *In silico* secondary structure prediction of 5'UTR+50bp in the coding sequence suggested a direct interaction between the T-rich region and the ribosomal binding site (data not shown). Synonymous mutations in this region (Mut_5-7) led to an overall decreased expression of NHBA, without affecting NHBA thermoregulation (Figure 3.2 C right panel).

A


MC58 wt
Pnhba
NMB2133
CREN
NHBA
NMB2131
% conservation
MC58 nhba intergenic region
-10
+1
-35
ACCGCTGCCGCCGATTTGGGCCGACAGAGGAACCGGGCGGAATAAACC
AAGCTATGCCGTCTGAAGCCCGTTTGGCGTTCAGACGGCATATTTTTA
CAATTTTATAGGCATACGGCGGAAACAATATTTATTGAATATGTTTCA
ACACACAGGGCGGCACATAAAGCACAGCCCTATGTGTCGCCCTGATTT
GGAAAGGTTGCGCTCTTCCCAAATAAAGCTGATTCCACCGCCCCGAAG
GACGGATGTCCAAGTGGCGGGGTTTAAACCAAAAAGGAAATACGATGA
AGGAGATGATGATGTTTAAACGCAGCGTAATCGCAATGGCTTGTATTT
TTGCCCTTTCAGCCTGCGGGGGCGGCGGTGGCGG

B


MC58 wt AAAGGAAATACGATGAAGGAGATGATGATGTTTAAACGCAGCGTAATCGCAATGGCTTGTATTTTTGCCCTTTCAGCCT
Mut_1 AAAGGAAATACGATGAAGGAG - - - ATGATGTTTAAACGCAGCGTAATCGCAATGGCTTGTATTTTTGCCCTTTCAGCCT
Mut_2 AAAGGAAATACGATGAAGGAG - - - - - - ATGTTTAAACGCAGCGTAATCGCAATGGCTTGTATTTTTGCCCTTTCAGCCT
Mut_3 AAAGGAAATACGCTGAAGGAGATGATGATGTTTAAACGCAGCGTAATCGCAATGGCTTGTATTTTTGCCCTTTCAGCCT
Mut_4 AAAGGAAATACGATGAAGGAGCTGCTGCTGTTTAAACGCAGCGTAATCGCAATGGCTTGTATTTTTGCCCTTTCAGCCT
Mut_5 AAAGGAAATACGATGAAGGAGATGATGATGTTTAAACGCAGCGTAATCGCAATGGCTTGTATCTTTGCCCTTTCAGCCT
Mut_6 AAAGGAAATACGATGAAGGAGATGATGATGTTTAAACGCAGCGTAATCGCAATGGCTTGTATTTTCGCCCTTTCAGCCT
Mut_7 AAAGGAAATACGATGAAGGAGATGATGATGTTTAAACGCAGCGTAATCGCAATGGCTTGTATCTTCGCCCTTTCAGCCT

C

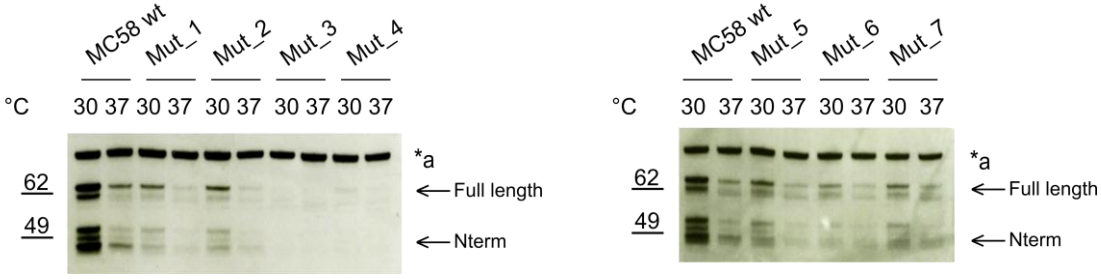
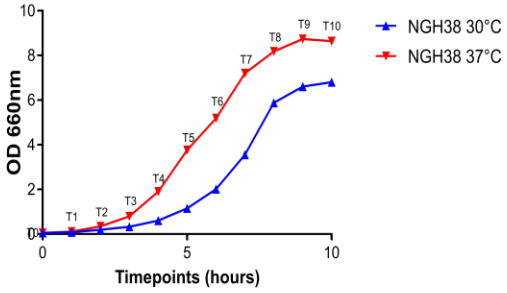

MC58 wt
Mut_1
Mut_2
Mut_3
Mut_4
°C
30 37 30 37 30 37 30 37 30 37
62
49
*a
Full length
Nterm
MC58 wt
Mut_5
Mut_6
Mut_7
°C
30 37 30 37 30 37 30 37
62
49
*a
Full length
Nterm

Figure 3.2 Schematic representation of *nhba* locus in MC58 strain and site-directed mutagenesis. (A) Schematic representation of the *nhba* locus (upper panel). The conservation of the indicated intergenic region obtained by multiple sequence alignments of 8373 *Neisseria* strains present in PubMLST database is shown (mid panel). Each bar represents the percentage of conservation of the corresponding nucleotide. Green bar = 100% identity; Greeny-brown bar < 100% identity; Red bar < 30% identity. The nucleotide sequence of the indicated region is reported (lower panel): the 3′ region of NMB2133 is boxed in dark grey and the 5′ region of *nhba* (NMB2132) is boxed in light grey. The nucleotides pairing in the stem region of the Rho-independent terminator are underlined (dot line). In the intergenic region downstream of NMB2133, a putative promoter sequence was identified (P*nhba*). The -35 and -10 elements of the P*nhba* are indicated and the putative transcriptional start site is indicated and highlighted in bold. The contact regulatory element of *Neisseria* (CREN), a 150-bp sequence specific for pathogenic *Neisseria* species, is present in MC58 strain immediately upstream of the ribosomal-binding site (Deghmane, A.E. et al. 2003) and is boxed in green. The ribosome binding sites are underlined and the translation start sites are boxed. **(B)** Schematic representation of site-directed mutagenesis. Red dashes indicate nucleotides deletion, red nucleotides indicate non-synonymous mutations (Mut_3 and Mut_4) and synonymous mutations (Mut_5-Mut_7). **(C)** Mutant strains were grown overnight on GC agar plates at the indicated temperatures. Whole cell lysates were prepared and separated by SDS-PAGE prior to Western Blotting. The indicated proteins were detected using mouse-polyclonal antisera. The *a symbol indicates a non-specific band used as loading control between different samples.

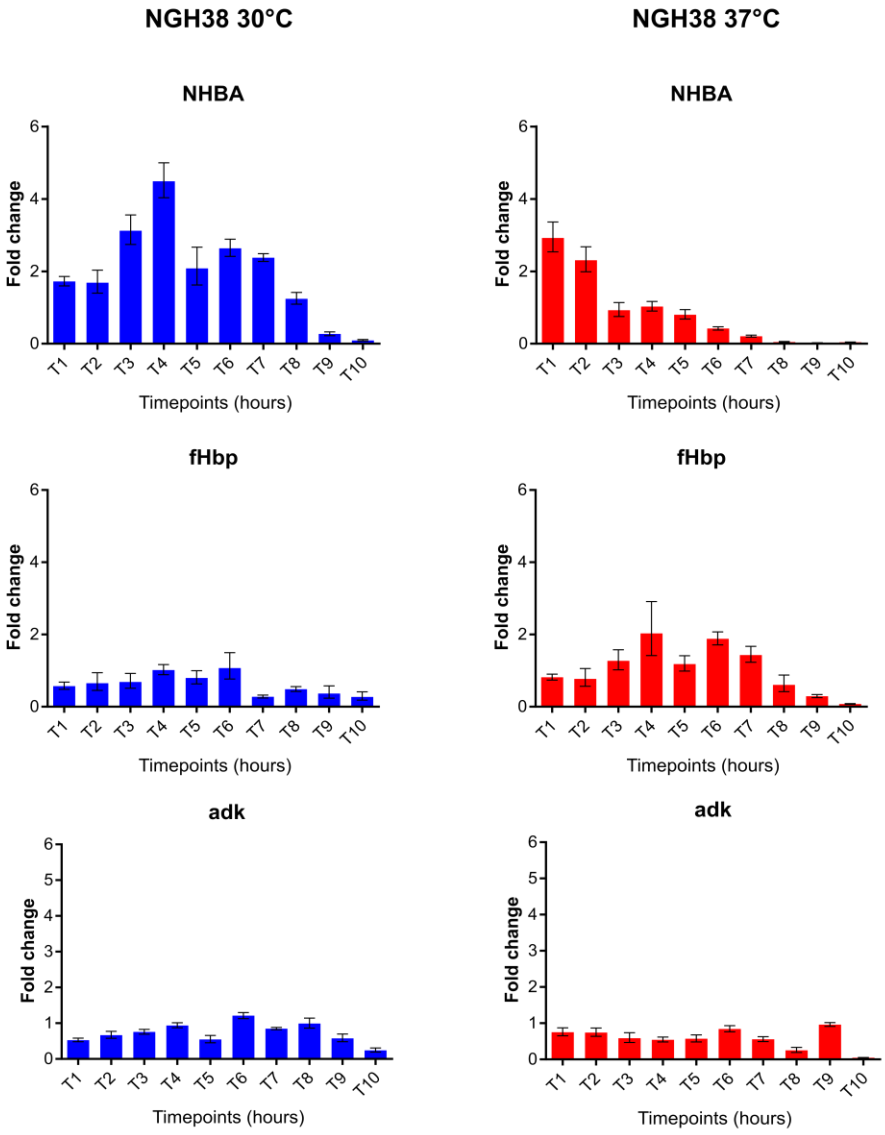
3.3. NHBA is expressed during the active growth

Previous analysis only represented a single time point of NHBA expression levels during mid-exponential growth. In order to determine how NHBA expression progresses during the entire growth of *N. meningitidis*, we grew NGH38 strain at either 30°C or 37°C in 50 ml of liquid culture. We took samples for RNA extraction and Western Blotting at various stages during the entire growth curve of the strain (Figure 3.3). By culturing the strain in flasks, we were able to take samples every hour and follow the growth for ten hours (Figure 3.3 A). Both curves reached high OD_{600} values however, under these experimental conditions, bacteria grown at 30°C showed longer lag phase (T0-T4) and slightly lower OD_{600} values at the end of the growth, respect to those grown at 37°C. Firstly, we investigated the transcriptional profile of *nhba, fHbp* and *adk* during growth in liquid culture (Figure 3.3 B). We determined by qRT-PCR that *nhba* transcript was most abundant during active bacterial replication. Once bacteria entered stationary phase, transcription of *nhba* was almost abolished. During the active replication, *nhba* RNA steady state levels were higher at 30°C respect to 37°C. Conversely, *fhbp* transcript resulted to be slightly more abundant at 37°C respect to 30°C, while no differences were observed for *adk* comparing the two temperatures. The *nhba* RNA steady state level expression profiles during the entire growth were confirmed also at protein level by Western blotting, showing that NHBA is most abundant during the active growth at both temperatures (Figure 3.3 C).The higher expression of NHBA also resulted in higher processing at 30°C.

A


OD 660nm
Timepoints (hours)
NGH38 30°C
NGH38 37°C
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10

B


NGH38 30°C
NGH38 37°C
NHBA
fHbp
adk
Fold change
Timepoints (hours)
T1 T2 T3 T4 T5 T6 T7 T8 T9 T10

C

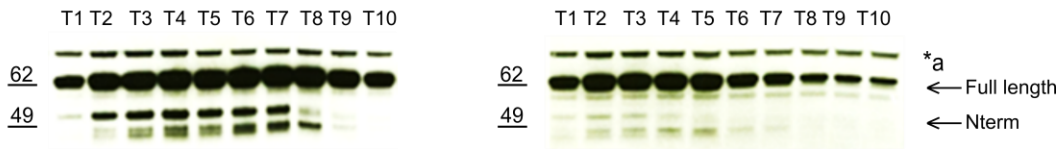

NGH38 30°C
NGH38 37°C
T1 T2 T3 T4 T5 T6 T7 T8 T9 T10
62
49
*a
Full length
Nterm

Figure 3.3 NGH38 growth profiles and NHBA expression levels during the entire growth. (A) Growth profiles of NGH38 strain in 50 ml of MCDMI liquid medium at 30°C (blu line) or 37°C (red line). Bacteria were grown for 10 hours and RNA isolation and samples for Western blot analysis were collected every hour. **(B)** *nhba*, *fHbp* and *adk* RNA steady state levels were quantified by qRT-PCR and relative expression levels were determined normalizing to *16S-rRNA*. **(C)** Western blot analysis of NHBA on whole cell lysates collected at the indicated time points. In NGH38 strain the full-length protein migrate with an apparent molecular weight of approximately 62 kDa, while other bands at approximately 49 kDa results from the bacterial proteases's processing (Serruto, D. et al. 2010). The *a symbol indicates a non-specific band used as loading control.

Therefore we decided to compare more in details the expression of NHBA in response to temperature by using standard *in vitro* conditions. We grew MC58 strain at either 30°C or 37°C in 7 ml liquid culture and took samples for RNA extraction and Western Blotting at various stages during the entire growth curve of the strain (Figure 3.4 A).
Firstly, we investigated the transcriptional profile of *nhba* during growth in liquid culture (Figure 3.4 B). By qRT-PCR we confirmed that *nhba* transcript was most abundant during active bacterial replication. Once bacteria entered stationary phase, transcription of *nhba* was almost abolished (Figure 3.4 B). Although there was a trend for slightly increased *nhba* transcript levels at 30°C, these transcriptional differences were only significant in late exponential phase. We therefore next examined the NHBA protein expression profile at the same growth phase as the *nhba* transcript levels. In accordance with the data for the *nhba* transcript, we observed that NHBA level at each temperature were highest during exponential growth of the bacterium (Figure 3.4 C). As seen previously, NHBA protein levels in each growth phase were always higher at 30°C relative to 37°C (Figure 3.4 C). However, although *nhba* transcript is barely detectable at stationary phase with no differences between the two temperatures, becomes evident that the protein is still abundant in bacteria cultured at 30°C. To quantify the differences in protein expression levels between the two temperatures tested, we used relative protein quantification (Figure 3.4 D). This analysis determined that the amount of NHBA at 30°C was approximately 3-5-fold higher than at 37°C.

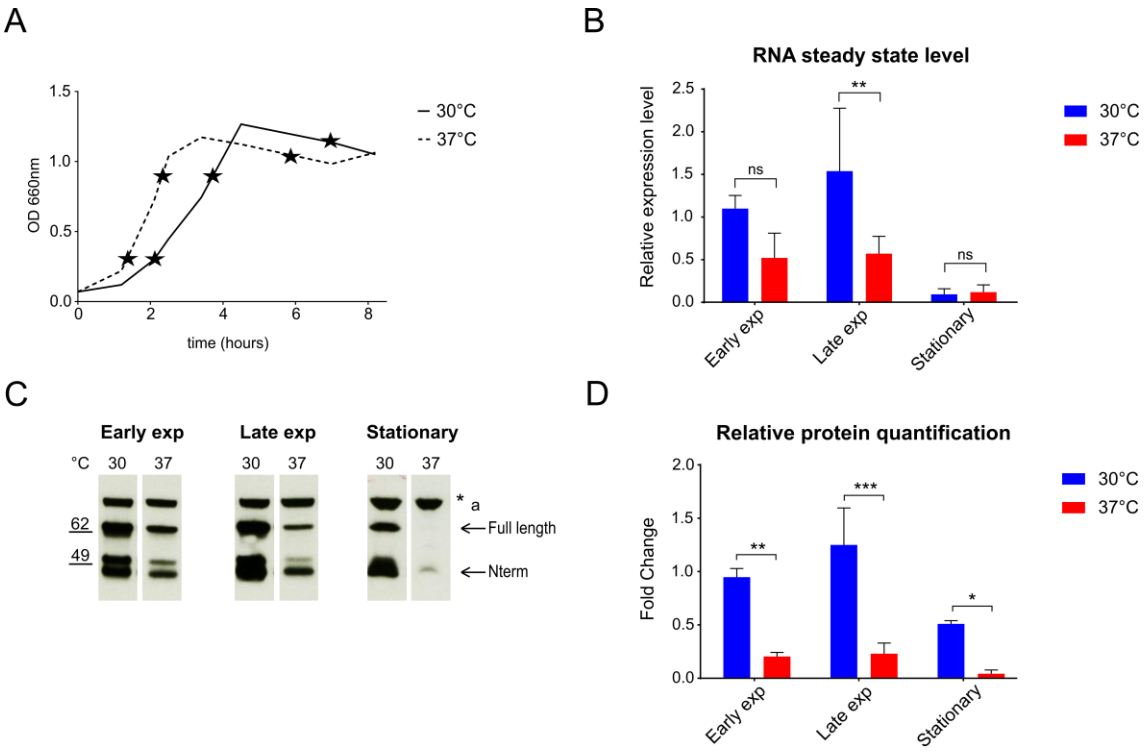


Figure 3.4 NHBA is expressed during the exponential phase and its expression at 30°C is higher relative to 37°C at both RNA and protein levels. (A) Growth profiles of MC58 strain in GC liquid medium at 30°C (continuous line) or 37°C (dot line). Samples for western blot analysis and RNA isolation were collected at early exponential phase (OD_{600} ~0.3), late exponential phase (OD_{600} ~0.9) and at stationary phase (OD_{600} ~1.1), as indicated by the star symbols. **(B)** *nhba* RNA steady state levels were quantified by qRT-PCR and relative expression levels were determined normalizing to *16S-rRNA*. **(C)** Western blot analysis of NHBA on whole cell lysates collected at the indicated time points. **(D)** Relative protein quantification performed with ImageJ 1.6 software. In MC58 strain the full-length protein migrate with an apparent molecular weight of approximately 62 kDa, while other bands at approximately 49 kDa results from the bacterial proteases's processing (Serruto, D. et al. 2010). The *a symbol indicates a non-specific band used as loading control and for relative protein quantification. All the data represent the mean +/- SEM from three independent biological replicates and were analyzed by Two-way Anova followed by uncorrected Fisher's LSD multiple comparison test (**** p<0.0001; *** p<0.001; ** p<0.01; * p<0.05; ns : not significant).

3.4. NHBA thermoregulation is not driven by the *nhba* promoter

To investigate the molecular mechanisms involved in NHBA thermoregulation a *nhba* deletion mutant (Δ*nhba*) was generated in the MC58 strain background by replacement with an erythromycin antibiotic resistance cassette and different isogenic complementation mutants were generated. To test if the genomic context played a role in *nhba* regulation, the complete sequence, comprising the entire gene and the intergenic regulatory region, was inserted into the NMB1428-NMB1429 genomic locus, generating the Δ*nhba*-C_*nhba* strain (Figure 3.5 A). As shown by Western blot analysis and qRT-PCR (Figure 3.6 A), the wild type and Δ*nhba*-C_*nhba* strains showed the same expression and thermoregulation of *nhba* as the wild-type strain, indicating that placing the wild type sequence of *nhba* in another genomic locus does not affect *nhba* regulation in response to temperature changes at both RNA and protein level.

In order to determine whether the *nhba* promoter was required for thermoregulation, we replaced the MC58 wild-type sequence with an IPTG-inducible Ptac promoter, immediately upstream of the CREN sequence (Figure 3.5 B), generating the Δ*nhba*-Ptac_*nhba* strain. We observed an IPTG dose-dependent increase of *nhba* expression at both RNA and protein level. However, using the same amount of IPTG for induction, we no longer observed any differences between the transcript levels at the two temperatures (Figure 3.6 B). Interestingly, we still observed clear thermoregulation of NHBA at protein level, albeit less pronounced than in the wild type strain.

To determine whether regulatory elements in the *nhba* upstream intergenic region contributed to thermoregulation, we fused the full intergenic region comprising the promoter, the CREN sequence and the initial part of the coding sequence corresponding to the first 14 amino acids to a *mCherry* reporter gene (Figure 3.5 C), generating the Δ*nhba*-Pwt_*mCherry* strain. As independent control, we also generated Δ*nhba*-Ptac_*mCherry* strain, a reporter fusion under the control of the IPTG-inducible promoter (Figure 3.5 D). We then analyzed the expression of the reporter gene product by qRT-PCR and Western blotting of samples collected at either 30°C or 37°C (Figure 3.6 C). The IPTG inducible promoter drove higher transcription of the downstream gene with respect to P*nhba* promoter. However, no significant differences were observed at protein level. We observed no thermoregulation in either reporter fusion construct, both at RNA and at protein level, suggesting that the promoter on its own cannot account for the observed differences in expression at the two temperatures. All

together these results highlight that NHBA thermoregulation is at the post-transcriptional level.

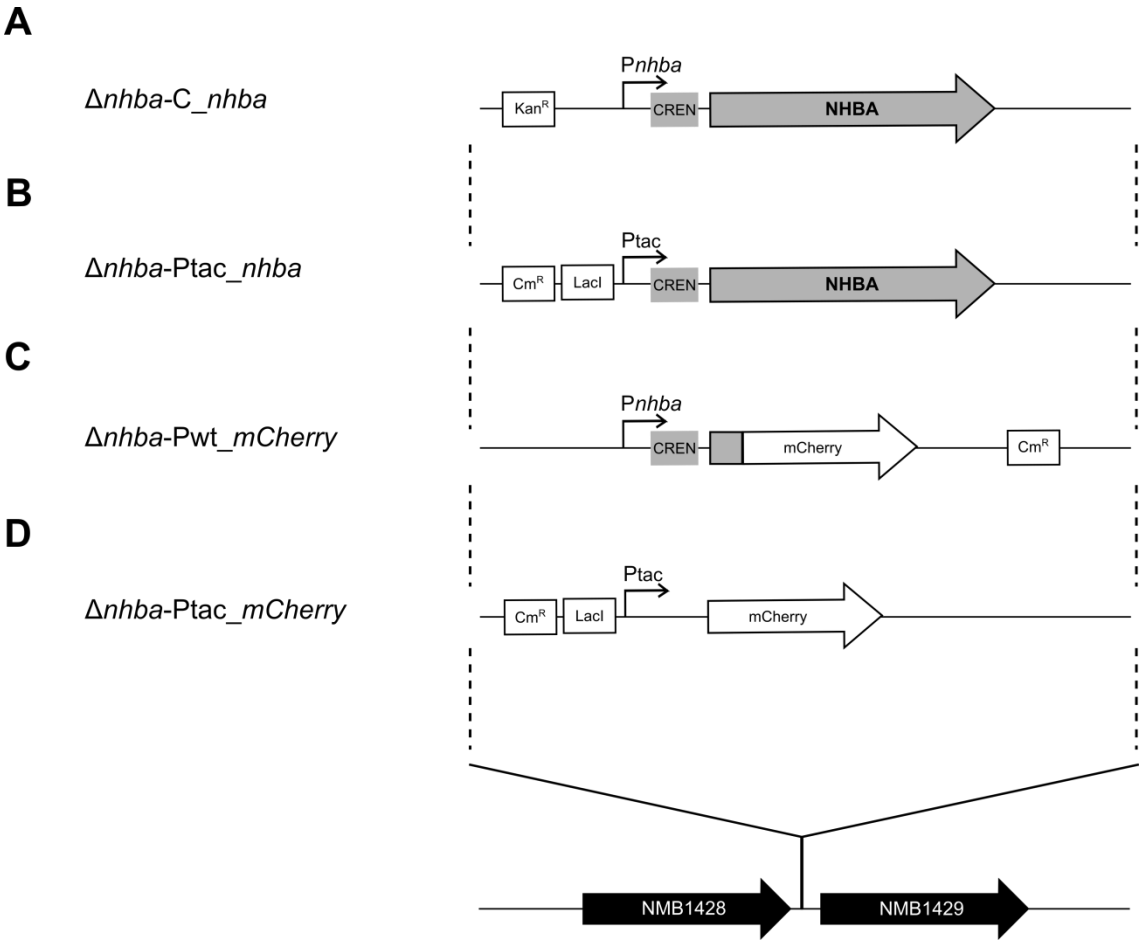


Figure 3.5 Schematic representation of *nhba* mutants generated by ex-locus complementation. (A-D) In the MC58Δ*nhba* strain background different mutants were generated by complementation in the NMB1428-1429 locus: Kan^R-kanamycin resistance cassette; Cm^R-chloramphenicol resistance cassette; LacI – LacI repressor gene; Ptac – IPTG inducible promoter; mCherry – mCherry reporter gene.

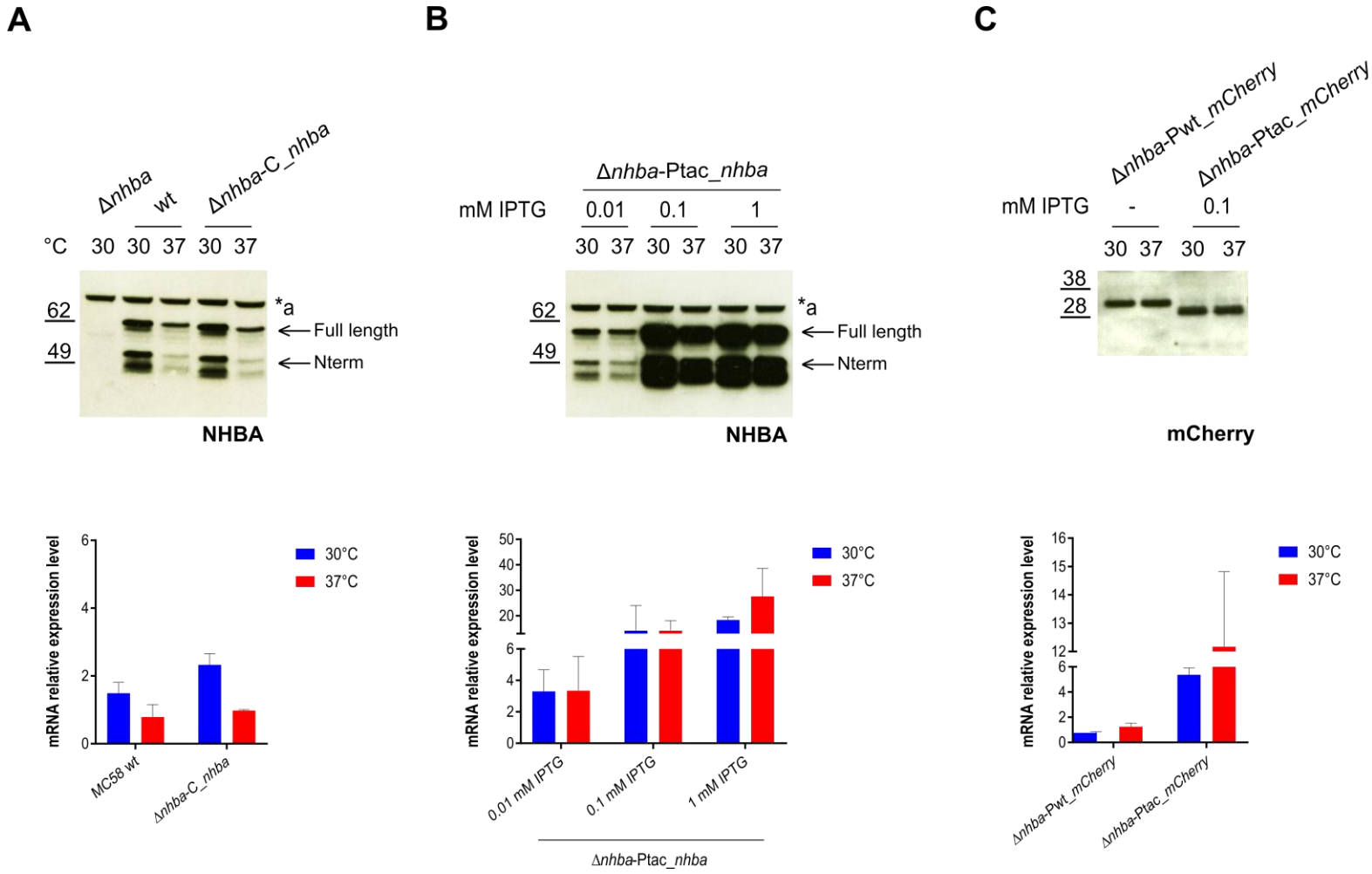


Figure 3.6 NHBA thermoregulation is at post-transcriptional level. (A-C) Wild type and recombinant strains were grown in GC liquid medium at 30°C or 37°C, with the indicated concentration of IPTG, where needed. NHBA and mCherry protein expression were assessed by western blotting by using polyclonal mouse antisera and monoclonal mouse antibody (ab167453, abcam), respectively (upper panel). *nhba* and *mCherry* RNA steady state levels were quantified by qRT-PCR and relative expression levels were determined normalizing to *16S-rRNA* (lower panel).

3.5. Temperature affects *nhba* RNA half-life

Transcription produces single-stranded RNA molecules that easily form intra- or intermolecular partially double-stranded RNAs, or associate with proteins, which may be used to regulate gene expression. To prevent or resolve kinetically trapped structures, cells use RNA chaperones that in most cases bind RNA non-specifically to help refold RNA or RNA–protein (RNP) complexes in ATP-independent or ATP-dependent reactions (Herschlag, D. 1995); (Mohr, S. et al. 2002); (Rajkowitsch, L. et al. 2007). The kinetic problem of RNA folding is dramatically aggravated at low temperatures. Few specific molecular mechanisms have been described to be involved in the cold shock response. CspA is the major regulator of the cold-shock response in *E.coli*, where the entire process of cold response is well documented. It is a RNA chaperone, able to bind both DNA and RNA, and its own regulation in response to temperature downshift is driven by a post-transcriptional mechanism which involves the 5'UTR region, resulting in higher transcript stability at low temperatures. The *N. meningitidis* homologue for cspA is NMB0838 and it resulted to be upregulated at 32°C in microarray analysis (data not shown). Another class of RNA binding proteins that accelerate structural rearrangements of RNA, particularly in the cold, are the DEAD-box RNA helicases. By using ATP as substrate, they mediate RNA conformational changes, otherwise kinetically unstable (Iost, I. et al. 2013). NMB1368 is a RNA helicase H present in *N. meningitidis*. Therefore, we decided to generate deletion mutants of NMB1368 and NMB0838 in MC58 strain background to investigate if this could have an effect on NHBA thermoregulation. Mutant strains did not show alterations in thermoregulation, both by qRT-PCR and Western blotting (Figure 3.7), even if a slightly decrease in expression was observed in ΔNMB0838 mutant grown at 30°C.

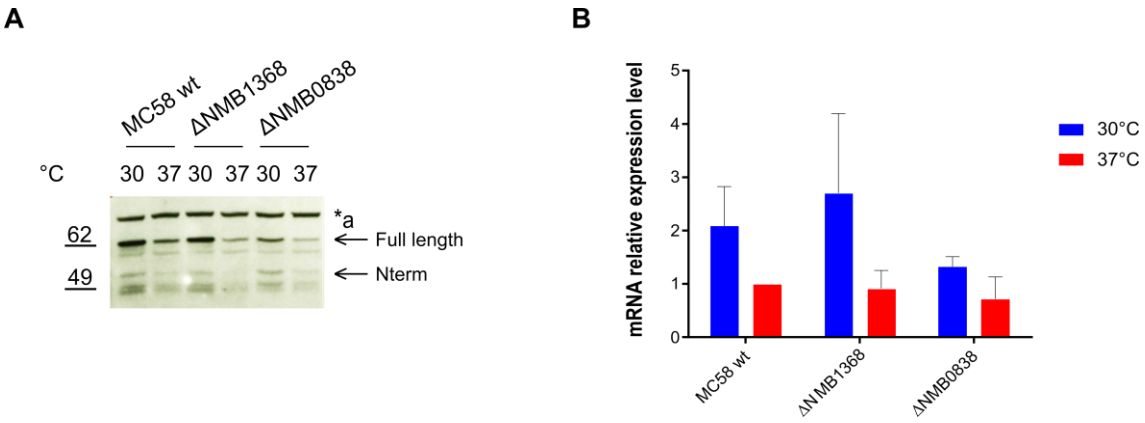


Figure 3.7 Deletion of NMB1368 or NMB0838 genes did not affect NHBA thermoregulation. NMB1368 or NMB0838 genes were deleted in the MC58 wt strain background. Mutant strains were grown in GC broth at 30°C or 37°C until OD_{600} 0.5. Whole cell lysates and samples for RNA isolation were collected. **(A)** Whole cell lysates were prepared and separated by SDS-PAGE prior to Western Blotting. The *a symbol indicates a non-specific band used as loading control and for relative protein quantification. **(B)** *nhba* RNA steady state levels were quantified by qRT-PCR and relative expression levels were determined normalizing to *16S-rRNA*.

We evaluated the *nhba* RNA decay after stopping active transcription by addition of rifampicin. The relative RNA amount was quantified by qRT-PCR (Figure 3.8). To obtain higher accuracy and reliability we used NGH38 as wild-type strain as overall *nhba* expression levels were higher compared to MC58. The transcript of *nhba* at 37°C showed a very short half-life and was rapidly degraded below the limit of detection (Figure 3.8). However, *nhba* transcript decay was found to be directly influenced by temperature, showing a shorter half-life at 37°C relative to 30°C

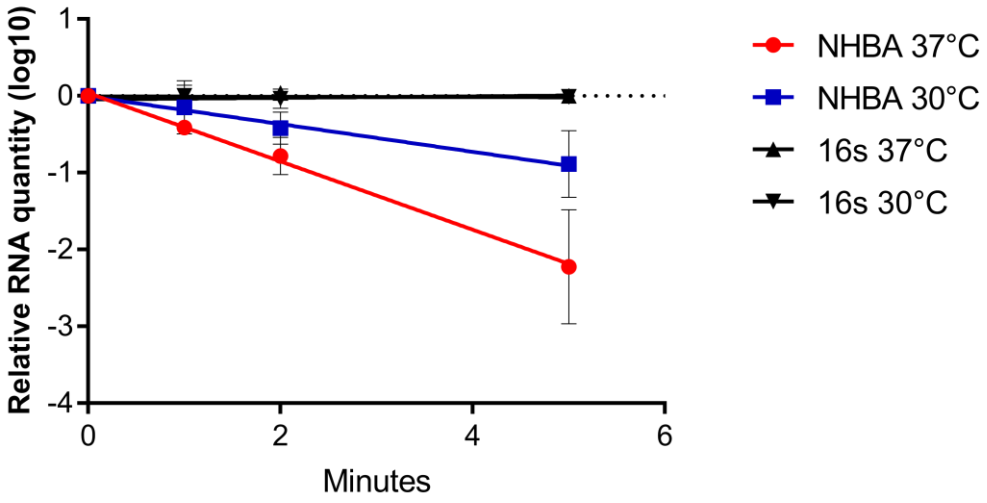


Figure 3.8 The ***nhba*** **transcript has a shortened half-life at elevated temperatures.** Strain NGH38 was grown in GC broth until OD_{600} 0.5 at the defined temperatures. RNA extracts were prepared at different time points after active transcription was stopped by adding rifampicin. *nhba* and 16s RNA abundance were measured by qRT-PCR and quantified relatively to the levels observed at the start of the experiment. Relative RNA quantity was calculated as $2^{-(Ct1-Ct0)}$ and transformed as Y= log(Y). A linear regression was performed for each dataset and plotted as continuous lines. Data represent the mean from three independent biological replicates ± SD.

3.6. NHBA protein shows higher stability at 30°C respect to 37°C

Having determined that NHBA protein levels are strongly affected post-transcriptionally, we wanted to investigate whether altered protein stability at different temperatures could account for the observed differences. We therefore grew the MC58 wild type strain in GC broth at 30°C and 37°C to mid-exponential phase and stopped protein translation by adding spectinomycin. Each culture was then split and incubated at both 30°C and 37°C and samples for whole cell protein extraction were withdrawn at different time-points of treatment. NHBA accumulation upon protein translation arrest was analyzed by Western blots with representative results shown in Figure 3.9. At 30°C, the amount of NHBA full length protein appears the same for 45-60 min treatment (Panel A, upper part), whereas treatment at 37 °C show no change for about 10 min treatment (Panel A, lower part).

Cells grown at 37 °C and treated with spectinomycin at 30 °C show a similar amount of NHBA for about 20 min treatment (Panel B, upper part), while treatment at 37 °C shows no changes for 10 min (Panel B, lower part). Overall, these data suggest that NHBA turnover is higher at 37°C and, accordingly, it appears stable at 30 °C. Thus, NHBA thermoregulation is additionally exerted by post-translational mechanisms.

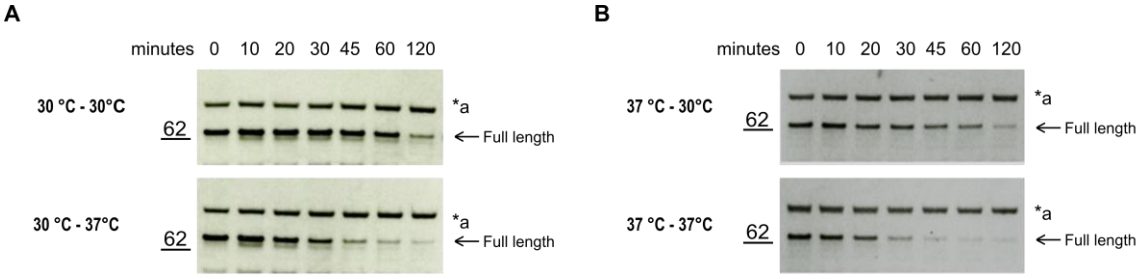


Figure 3.9 NHBA protein turnover is directly affected by temperature changes. (A-B) MC58 wild type strain was grown in GC broth until OD_{600} 0.5 at 30°C and 37°C. Whole cell extracts were prepared at different timepoints after the active translation was stopped by adding spectinomycin. Protein samples were separated by SDS-PAGE prior to Western Blotting using a anti-NHBA mouse polyclonal serum. The full length protein is shown. The *a symbol indicates a non-specific band used as loading control.

3.7. NHBA expression levels correlate with susceptibility to complement-mediated killing by anti-NHBA antibodies

NHBA is one of the three major components of the 4CMen vaccine against serogroup B meningococcus and is a protective antigen that is able to elicit a robust immune response. Although NHBA is present in all neisserial species, we have shown that its expression is variable among strains and moreover is affected by several factors such as like growth phase and temperature changes. It is therefore paramount to understand how different NHBA expression levels, either through strain variation or triggered by different growth conditions affect the bacterium's susceptibility to killing mediated by anti-NHBA antibodies.

We therefore investigated whether the different NHBA expression levels at different temperatures could affect bacterial susceptibility to complement-mediated killing. To address this hypothesis, we grew strain MC58 at both 30°C and 37°C and confirmed increased NHBA and decreased fHbp expression levels at 30°C compared to 37°C (Figure 3.10 A). Functional antigen should be exposed on the bacterial cell surface and we verified that altered antigen expression levels translated into altered surface exposure looking at NHBA, fHbp and cps surface exposure by flow cytometry (Figure 3.10 B). Given these observations, we assessed the ability of immune serum raised against NHBA to kill *N. meningitidis* grown at the two different temperatures by determining their serum bactericidal titer (Figure 3.10 C). This assay is also called serum bactericidal assay (SBA) and was performed at both 30°C and 37°C. While clear differences in antigen expression were evident by Western blotting and flow cytometry analysis and trends of the SBA assays reflected these differences, we were unable to gain statistical significance due to the intrinsic variability of the experiment. We reasoned that altering the temperature would affect many different processes in the bacterial cell and confounding pleotropic effects would make it difficult to determine the precise impact of NHBA expression levels on serum bactericidal killing. For example, important virulence factors such as fHbp and the neisserial capsule also respond to temperature changes, but in the opposite way compared to NHBA (Figure 3.10 B). These pleiotropic effects could then mask the role of NHBA in this assay making it impossible to extract how NHBA expression levels affect bacterial killing in this assay.

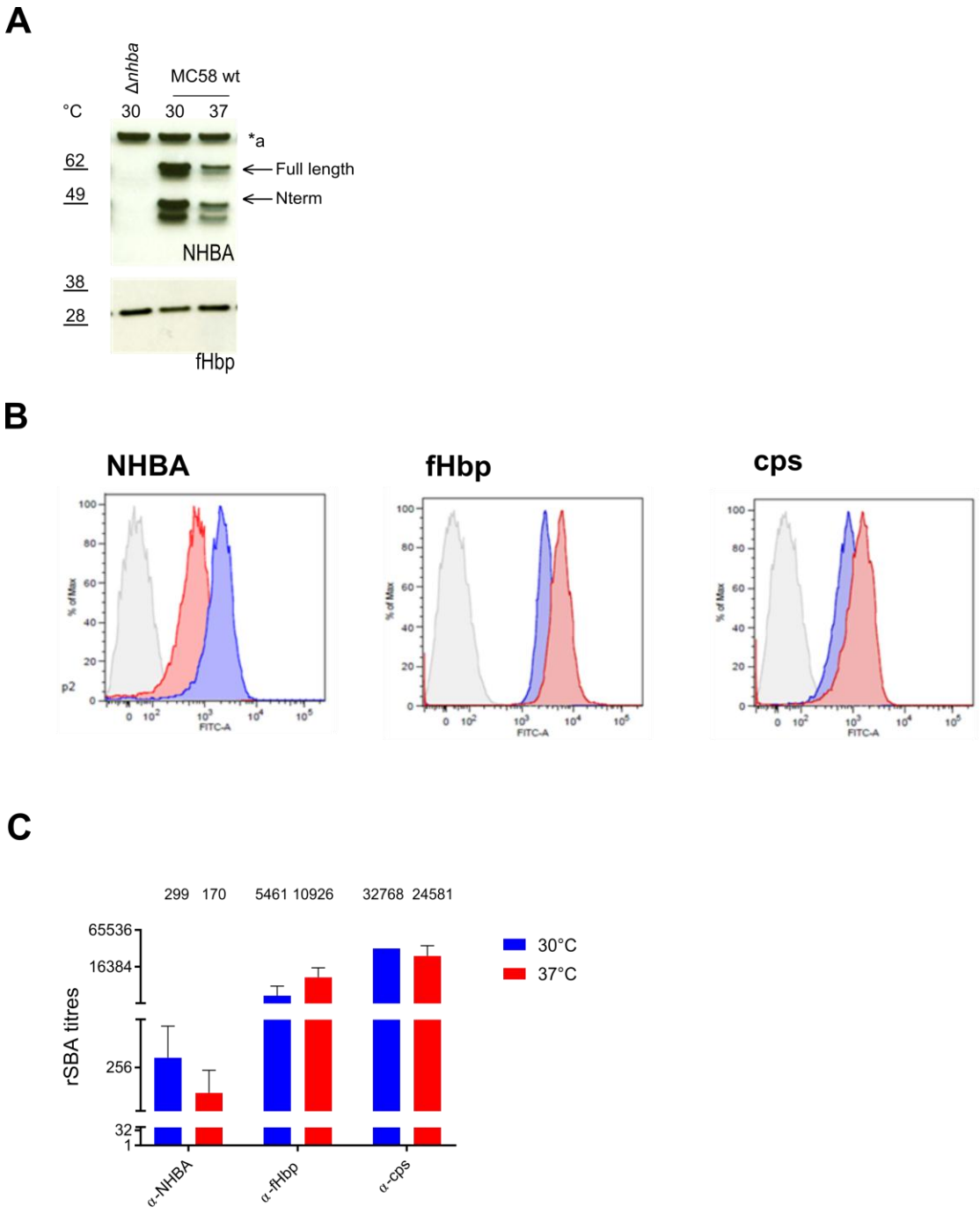


Figure 3.10 Temperature driven expression, surface exposure and susceptibility to complement-mediated killing. The MC58 wild type strain was grown at 30°C or 37°C in MH broth +0.25% glucose until OD600 0.25 was reached. Bacteria were collected and expression levels of NHBA and fHbp were determined by **(A)** Western blotting and surface exposure of the defined antigens was confirmed by **(B)** flow cytometry using polyclonal antisera. **(C)** Serum bactericidal titers were determined using baby rabbit complement as source of complement factors (rabbit SBA, rSBA). rSBA titers indicate the dilution of the α-NHBA mouse polyclonal serum, α-fHbp mouse polyclonal serum or α-cps mouse monoclonal antibody at which 50% of killing was reached. All the data are representative of three independent biological replicates.

In order to circumvent pleiotropic effect on bacterial expression of virulence factors other than NHBA, we generated a recombinant strain in which NHBA expression was under the control of an IPTG-inducible promoter (MC58 Δ*nhba*-Ptac_*nhba*). This assay allowed us to perform the experiment under identical conditions while varying NHBA expression through addition of different IPTG concentrations. We confirmed protein expression and surface exposure of NHBA in these cultures using Western blotting and flow cytometry analysis (Figure 3.11 A and B, respectively).This then enabled us to extract the role of NHBA expression levels in the ability of anti-NHBA antiserum from mice to mediate complement-dependent killing through the rSBA (Figure 3.11 C). We observed that rSBA titers correlated directly with NHBA expression levels, as confirmed by statistical analysis.

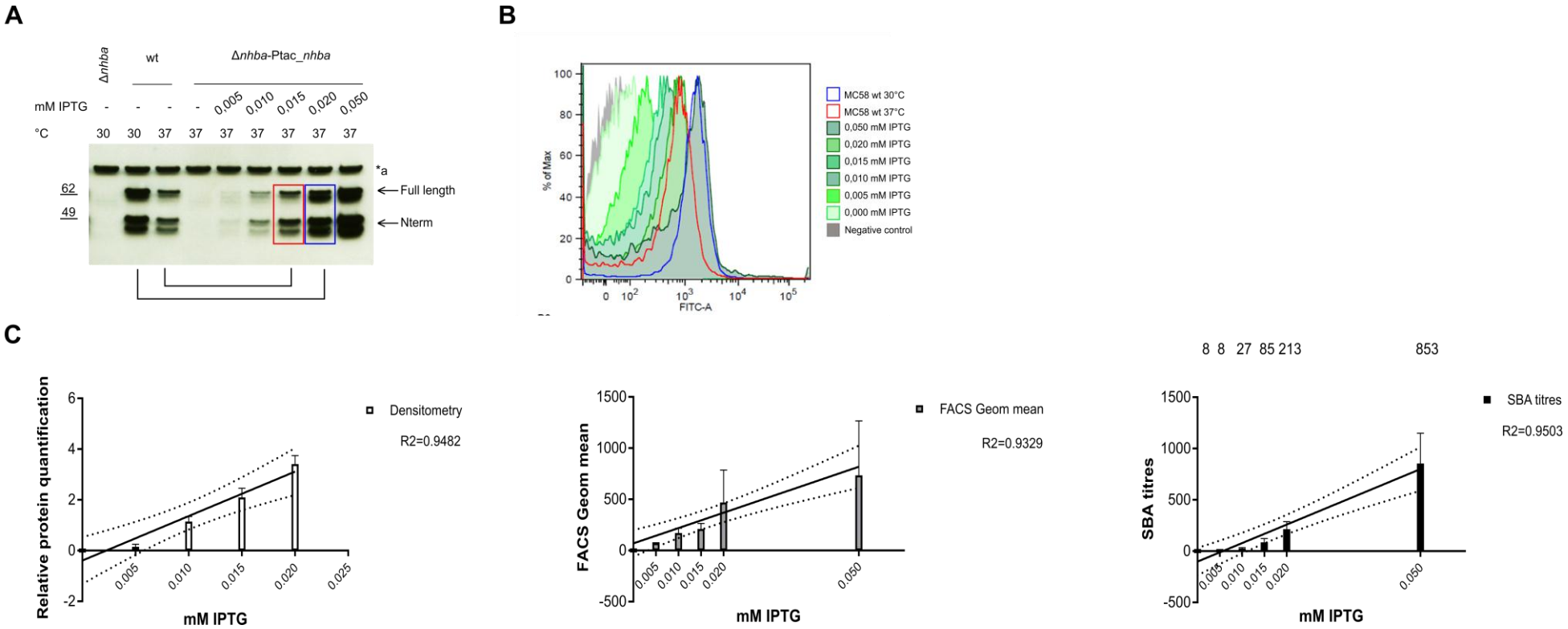


Figure 3.11 Correlation between NHBA expression, surface exposure and susceptibility to complement-mediated killing by anti-NHBA antibodies. MC58 Δ*nhba*, wild type and Δ*nhba*-Ptac_*nhba* strains were grown in MH broth +0.25% glucose until OD_{600} 0.25 at 30°C or 37°C as indicated. IPTG was added, where needed, at the indicated final concentrations. Bacteria were collected to perform **(A)** Western blotting, **(B)** flow cytometry analysis and **(C)** serum bactericidal assay. The relative quantification obtained by **(A)** densitometry analysis and **(B)** FACS geometric mean calculation were estimated. All the data represent the mean +/- SD from three independent biological replicates and a linear regression was performed on each dataset (black lines indicate the linear fit, dot lines indicate the 95% CI). A Pearson correlation test was used to assess the goodness of correlation between the three different datasets (Densitometry/SBA titers: Pearson r = 0,962; P=0.009.

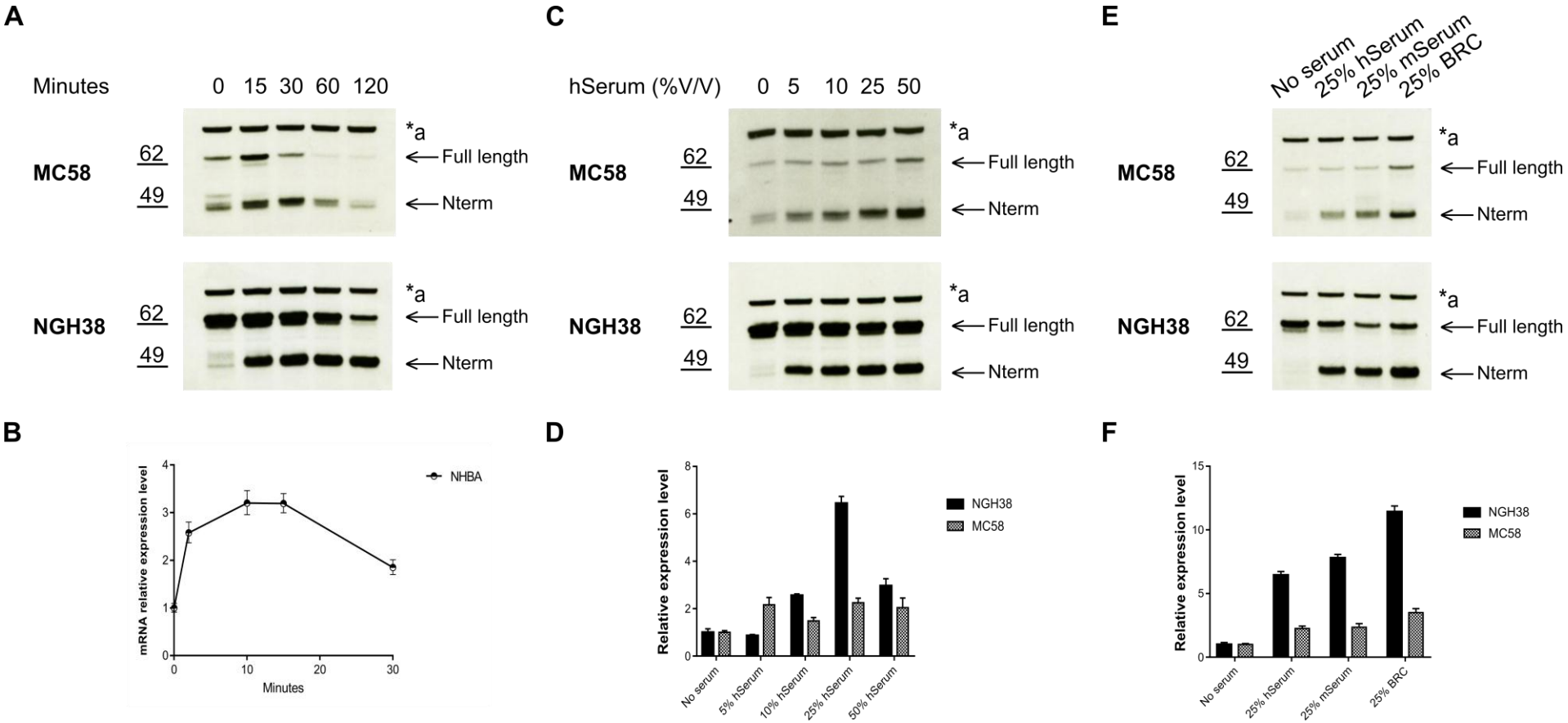
Densitometry/FACS GeoMean: Pearson r = 0,972; P=0.006; FACS GeoMean/SBA titers: Pearson r = 0,925; P=0.008). Western blotting relative quantification of 0.050 mM IPTG samples were not taken into account for the linear regression as these were found to be out of the linearity range of the assay

3.8. NHBA regulation during invasion

It has been previously shown that NHBA can be processed by the meningococcal protease NalP and human lactoferrin. Cleavage occurs either upstream and downstream of the NHBA Arg-rich region resulting in one of two possible cleavage fragments termed C2 and C1, respectively (Serruto, D. et al. 2010). To mimic the invasive condition MC58 and NGH38 wild type strains were grown until early exponential phase and then incubated at 37°C in presence of 25% of human serum during a time course, up to 2 hours (Figure 3.12 A and B). After 15 minutes of serum incubation NHBA was induced and strongly processed in both strains. In fact, despite the appearance of the lower bands typical of the N terminal portion of NHBA after cleavage of the protein, the band corresponding to the full length protein appeared to retain the same intensity or even more suggesting induction of the full length NHBA as well as concomitant processing. Within the blood there is an abundance of components with proteolytic activity, so it is not surprising to see an increase in cleavage. The induction and the processing proceed for the first 30 minutes after which, under these in vitro conditions, the overall amount of the full length protein decreases strongly with different kinetics among the two strains. Not only they express different levels of *nhba*, moreover the different background is somehow responsible for different stability of the protein. Therefore, while in MC58 the band corresponding to the full length protein almost disappeared after 2 hours of incubation, in NGH38 strain it is only slightly reduced. The induction is confirmed also at the transcriptional level, as shown by qRT-PCR (Figure 3.12 B). Following serum incubation a transient induction of transcriptional induction was immediately registered, with maximal levels after 30 minutes.

We assessed the serum dose-response by incubating both strains at 37°C for 30 minutes in a dose-range of human serum. Even at the lowest concentration of serum, cleavage of NHBA is observed in both strains. A proportional increase in the processed band, correlated with serum dose, was clearly observed in MC58 strain and less apparent in NGH38 strain (Figure 3.12 C). Although serum induction is confirmed at the RNA level (Figure 3.12 D), a direct dose-response correlation was not observed. This is maybe due to pleiotropic effects of serum bactericidal killing on total mRNA levels. Finally, we compared the effects of different sources of serum on NHBA expression. Both

induction and cleavage events are triggered by mouse, human and baby rabbit serum sources, even to slightly different extent (Figure 3.12 E and F).


A
Minutes
0 15 30 60 120
MC58
62
49
*a
Full length
Nterm
NGH38
62
49
*a
Full length
Nterm
B
mRNA relative expression level
NHBA
Minutes
C
hSerum (%V/V)
0 5 10 25 50
MC58
62
49
*a
Full length
Nterm
NGH38
62
49
*a
Full length
Nterm
D
Relative expression level
NGH38
MC58
No serum
5% hSerum
10% hSerum
25% hSerum
50% hSerum
E
No serum
25% hSerum
25% mSerum
25% BRC
MC58
62
49
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Full length
Nterm
NGH38
62
49
*a
Full length
Nterm
F
Relative expression level
NGH38
MC58
No serum
25% hSerum
25% mSerum
25% BRC

Figure 3.12 NHBA protein and RNA expression levels after serum incubation: time course, dose-response and different sources of serum. (A) MC58 wt and NGH38 wt strains were grown at 37°C in MH broth +0.25% glucose until OD_{600} 0.25 was reached. Bacteria were collected and incubated at 37°C in DPBS++ with 0,1% glucose, +1%BSA and 25% of human serum. Whole cell lysates were prepared at the indicated timepoints. Protein samples were separated by SDS Page prior to Western blotting. **(B)** RNA extracts (NGH38 wt strain) at the indicated time points were prepared. *nhba* RNA steady state levels were quantified by qRT-PCR and relative expression levels were determined normalizing to *16S-rRNA* **(C-D)** MC58 wt and NGH38 wt strains were grown at 37°C in MH broth +0.25% glucose until OD_{600} 0.25 was reached. Bacteria were collected and incubated for 30 min at 37°C in DPBS++ with 0,1% glucose, +1%BSA and different concentration of human serum. **(C)** Whole cell lysates were prepared and analysed by Western blotting. **(D)** RNA extracts were performed in parallel and *nhba* RNA steady state level was determined by qRT-PCR **(E-F)** MC58 wt and NGH38 wt strains were grown at 37°C in MH broth +0.25% glucose until OD_{600} 0.25 was reached. Bacteria were collected and incubated for 30 min at 37°C in DPBS++ with 0,1% glucose, +1%BSA, in presence or not of 25% of human serum (hSer), mouse serum (mSer) or baby rabbit complement (BRC). Western blotting and qRT-PCR analysis were performed. The *a symbol indicates a non-specific band used as loading control between different samples.

3.9. NHBA cleavage does not affect NHBA-mediated killing susceptibility

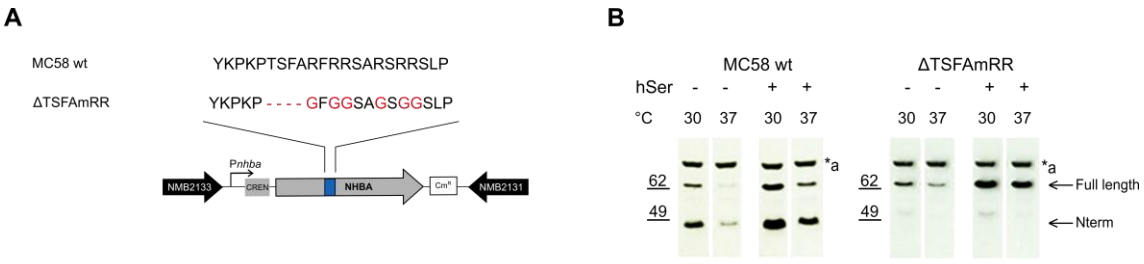
Previously, it was shown that mutation of the arginine stretch abrogates cleavage by lactoferrin but was not sufficient to block NalP cleavage (Serruto, D. et al. 2010). NalP cleaves at serine residue directly upstream of the Arg-rich region. Mutation of this serine alone into an alanine by site directed mutagenesis did not abrogate NalP cleavage (data not shown). At least eleven putative NalP target domains have been identified through a bioinformatics approach (Muzzi, A. et al. 2013, Serruto, D. et al. 2010) within different NHBA peptides. In particular it was observed that NHBA peptide p5 seems to lack the residues which are usually recognized by NalP (Table 3.2) and this peptide is resistant to NalP cleavage (Vacca, I. 2014).

NalP target domain name	NalP target domain sequence	NHBA variant and isoform
NalP_td_1	YKPKP **- - TSFA**RFRRSA	p0001 (Long)
		p0002 (Long)
		p0003 (Long)
NalP_td_2	YKDKS **- - TSSA**QFRRSA	p0017 (Short)
		p0018 (Short)
NalP_td_5	YKDK**SASSSFA**RFRRSA	p0021 (Short)
NalP_td_6	YKDK**SASSSSA**RFRRSA	p0020 (Short)
		p0029 (Long)
NalP_td_8	YKPKT **- - TSSA**RFRRSA	p0010 (Short)
NalP_td_11	YTDKP**- - - - - - - -**PTRSA	p0005 (Long)

Table 3.2 Analysis of NalP target domain sequences. Six different classes of NalP target domains were observed for NHBA peptides p1, p2, p3, p5, p10, p17, p18, p20, p21, p29. These classes are indicated in the first column of the table. The last column shows an example of NHBA peptides associated to the different NalP target domains. (Adapted from (Vacca, I. 2014))

Based on this observation we generated a MC58 recombinant strain called ΔTSFAmRR, expressing a NHBA protein in which we combined the deletion of the NalP target domain sequence TSFA with the mutation of the Arg-rich domain in a Gly-stretch (Figure 3.13 A). Respect to the wild type, ΔTSFAmRR strain resulted to be fully resistant to cleavage by NalP and other proteases, even in presence of serum (Figure 3.13 B). When testing the thermoregulation of NHBA expressed in the mutant strain, even if less pronounced, differences in protein amount at 30°C and 37°C were still

visible. We tested whether the resistance to cleavage would affect the susceptibility of MC58 to anti-NHBA antibodies in a serum bactericidal assay. The strains were grown for the bactericidal assay at 30 and 37°C. A moderate difference of one titre, as shown previously (Figure 3.10 C), was observed in response to temperature for both the wild type and the ΔTSFAmRR strains (Figure 3.13 C). However, no differences were observed in bactericidal titres between the wild type and the recombinant strain by using polyclonal mouse serum against the full length protein indicating that the cleavage of NHBA does not affect the overall bactericidal susceptibility of the strain to anti-NHBA antibodies. By using a polyclonal mouse serum specific for the C2 fragment of the protein, only the ΔTSFAmRR strain at 30°C showed a barely positive SBA titer.



C

	MC58 wt 30°C	MC58 wt 37°C	ΔTSFAmRR 30°C	ΔTSFAmRR 37°C
α-NHBA FL	2048	1024	2048	1024
α-NHBA C2	<4	<4	8	<4

Figure 3.13 Expression and killing susceptibility of MC58 wild type and ΔTSFAmRR mutant strains. (A) ΔTSFAmRR mutant was generated by two step of site-directed mutagenesis within the *nhba* sequence. The mutated *nhba* sequence was used for in locus complementation in the MC58Δ*nhba* strain background. The wild type and mutant strains were grown in in MH broth +0.25% glucose until an OD_{600} of 0.25 at 30°C or 37°C, as indicated. Bacteria were collected before and following the incubation in presence of 25% of baby rabbit complement at 37°C for 1 hour and total protein extracts were performed. **(B)** Western blotting and **(C)** serum bactericidal assay. The rSBA was performed at the indicated temperatures, using two polyclonal mouse sera, one specific for the C-terminal domain of NHBA (α-NHBA C2), and one able to recognize the full length protein (α-NHBA FL).

4. Discussion and conclusions

The meningococcus has successfully evolved to survive in the human nasopharynx, which is its only natural habitat. Colonization with this bacterium is frequent especially in young adults, however establishing a successful colonization is a very challenging step. Within the nasopharynx there are several temperature gradients. For example, the temperature on the surface of the anterior nares is around 30°C at the end of inspiration, and rises to around 34°C in the posterior nasopharynx and tonsillar region (Keck, T. et al. 2000). Both these sites on the mucosal surface are significantly cooler than the core body temperature of 37°C, where the bacterium replicates during invasive disease. Additionally, fluctuations in the local temperature will be generated by acute inflammation, resulting in increased blood flow and systemic illnesses (Eccles, R. 2005). Even slight temperature differences have a significant impact on the phenotype and proteome of *N. meningitidis*. Adhesive properties of *N. meningitidis* appear stronger at 32°C in comparison to 37°C and recently another study reported also that one of the most induced protein at low temperature resulted to be NHBA (Lappann, M. et al. 2016). NHBA is an important virulence factor for *N. meningitidis*, being involved in adhesion (Vacca, I. et al. 2016), biofilm formation (Arenas, J. et al. 2013) and possibly even in serum resistance through its ability to bind heparin (Serruto, D. et al. 2010) (Esposito, V. et al. 2011). It is also a protective antigen able to elicit an immune response in preclinical and clinical testing (Serruto, D. et al. 2010), therefore understanding the mechanisms that drive NHBA regulation is an important goal to better understand *N. meningitidis* pathogenesis and vaccine induced response.

We decided to investigate how physiologically relevant temperatures, which mimic the different stages of pathogenesis, may affect its expression. In particular, we found that NHBA expression was highly induced at 30°C, similar to what was shown by a comparative proteomic study recently reported (Lappann, M. et al. 2016). Although expressed in different variants and isoforms, NHBA is a lipoprotein presents in all *Neisseria* species.

We showed that NHBA expression is at its highest level between 28°C and 30°C, also corresponding to higher surface exposure. The regulation in response to temperature is driven by a mechanism conserved among all the sixteen different strains tested,

irrespective of the clonal complex, the NHBA variant and/or the isoform expressed, suggesting the relevance of NHBA regulation for the bacterial physiology.

Multiple sequence alignment of the intergenic and the 5'TR region of *nhba* among 8373 sequences present in the PubMLST database showed a high conservation among *Neisseria* species. As exception, the CREN sequence is present only in hypervirulent strains belonging to ST-32 (i.e. MC58). It has been shown that the CREN sequence mediates the overexpression of downstream gene in response to cell contact (Deghmane, A.E. et al. 2003). When present, the CREN sequence promotes *nhba* expression, again suggesting the importance of a strictly regulated NHBA expression during host-pathogen interaction. Nevertheless, the molecular mechanism that drives this regulation is not yet fully understood. However, i*n vitro* evidence shows that Hfq directly bind RNA-CREN region upstream to *nhba* (Antunes A., personal communications). Hfq has been reported to modulate the half-life of some mRNAs directly or by stimulating their poly(A) adenylation (Brennan, R.G. et al. 2007). These observations suggested that the 5'UTR can stabilize the messenger against ribonucleases. This would be achieved by hairpin structures, RNA binding proteins and by sequestered ribosomes, and it can be hypothesized that a putative host factor, or a bacterial factor induced by the host, binds to the UTR and thereby controls the initiation of translation of the virulence gene. However the mechanism behind cell-contact upregulation driven by the CREN sequence does not concern the mechanism behind thermoregulation, since even strains that lack the CREN sequence show fluctuations in NHBA expression in response to temperature. Nevertheless, the *nhba* 5'UTR resulted to be a very sensitive region for its expression, anyway. Small deletions or even single-base mutations directed to the different putative translational start sites identified within this region affect *nhba* expression even though they do not impair thermoregulation. *In silico* RNA secondary structure predictions revealed that a U-rich region identified in the 5' of the mRNA may base-pair to the RBS, suggesting a possible effect on RNA translatability. Synonymous mutations within this tract, where even just a single base was replaced, have a dramatic effect on the overall NHBA expression, despite no direct effect on thermoregulation was observed. Taking together these results highlight the crucial role and the sensitivity of the 5'UTR and the 5'TR for NHBA expression.

We determined that NHBA expression is growth-phase dependent, with the maximum of expression reached during active replication. The growth related mechanism(s), acts maybe at transcription level, since the RNA is almost undetectable when stationary phase is reached. The reasons why this gene is shut-down at stationary phase have not been clarified yet. However, at 30°C the protein resulted to be still abundant even at stationary phase, while no bands were visible in extracts from bacteria grown at 37°C until stationary phase, suggesting differences in protein stability between 30°C and 37°C. NHBA expression resulted to be higher at 30°C in each growth phase. These observations led to the consideration that the regulations in response to temperature changes and growth phase are not related, and moreover underline that *nhba* is a very highly regulated gene with different mechanisms that respond to different conditions and stimuli. Nevertheless, by *in silico* prediction no transcription factor binding sites have been found within the *nhba* intergenic region.

Our results revealed that multiple molecular mechanisms are responsible for the overall regulation of *nhba* in response to temperature changes. We excluded that the genomic context plays a role in this regulation, since replacing the wild type gene in another genomic locus does not affect the response to temperature. However, the upstream intergenic region *per se* is not sufficient to drive the response to temperature changes, in fact by fusing it to a *mCherry* reporter gene thermoregulation is lost. However, by replacing the wild type promoter with an IPTG-inducible one, differences at RNA steady state level are lost, while are still present at protein level even if less pronounced than in the wild type strain. This suggest that somehow the wild type promoter is requested to drive the regulation in response to temperature detectable at RNA level, but moreover highlights that post-transcriptional and post translational mechanisms are involved. The upstream regulatory sequences *per se* are required but not sufficient to obtain the differences observed at RNA steady state level. Instead of a direct role in generating differences by alterations in active transcription, the wild type upstream sequences seems to be involved somehow in affecting *nhba* mRNA stability and therefore acting at post-transcriptional level. Lapann and colleagues hypothesized, but excluded a direct role of σ^E factor (Lappann, M. et al. 2016). Most likely the 5′ UTR and possibly 5′ coding region are required for the formation of secondary structures that act as a switch in regulating RNA stability, probably affecting the availability of sites recognized by RNA binding proteins or ribosomes recruiting.

RNA thermosensors are an energetically efficient strategy that offers rapid responses to abrupt changes in the temperature, as seen during the onset of inflammation. RNA thermometers respond to the absolute temperature as a physical parameter. They have evolved to sense and translate ambient temperature signals to the translation machinery in an immediate manner since they control the translation of already existing or nascent mRNAs. Therefore, temperature changes have an instantaneous effect on the expression of the RNA thermometer-associated transcript and allow a rapid and cost-effective response. RNA thermometers are precisely calibrated sensory devices that can detect temperature variations on the 1 °C scale (Kortmann, J. et al. 2012). Typical RNA thermometers control translation initiation by forming a secondary structure that entirely or partially includes the 5'UTR of an mRNA, modulating the translation efficiency through a thermodynamic-driven mechanism. All three major temperature-responsive gene classes — virulence genes, heat shock genes and cold shock genes — exploit this concept, making use of RNA thermometers to control translation initiation in a temperature-dependent manner (Narberhaus, F. 2010, Narberhaus, F. et al. 2006). Usually, the structure traps the RBS and the increase in temperature destabilizes it and permits formation of the translation initiation complex. Evidences have been reported recently for fHbp and capsule regulation in *N. meningitidis* (Loh, E. et al. 2013, Loh, E. et al. 2016). However, a zipper-like mechanism is clearly not compatible with responses that activate gene expression at low temperatures.

Acclimation to low temperatures requires the production of several cold shock proteins that counteract membrane rigidification, as well as the formation of stable RNA structures that interfere with translation initiation and elongation (Kortmann, J. and Narberhaus, F. 2012).

One of the best characterized regulation in response to cold shock is the massive induction of the expression of *E.coli* cold shock protein A (CspA), an RNA chaperone that binds single-stranded RNAs to prevent formation of secondary structures in cold temperatures (Jiang, W. et al. 1997). The cspA transcript is very unstable at 37 °C, but is dramatically stabilized by global reorganization of its architecture following a temperature downshift to 10 °C (Yamanaka, K. et al. 1999). In addition, translational control by an in built RNA-based thermosensor enhances cspA expression at low temperatures. Deletion analysis of the 160 nucleotide 5′ UTR of cspA indicated that

cold induction is mainly regulated at the post-transcriptional level by cis-acting elements in the transcript (Yamanaka, K. et al. 1999). The 'cold shock structure' is more efficiently translated and somewhat less susceptible to degradation than the structure at 37 °C (Giuliodori, A.M. et al. 2010). The thermosensory region extends far into the cspA coding region, and the RNA structure depends on a kinetically driven RNA-folding process. Sixty nucleotides of the coding region are needed for the decision making process and there is only a single hairpin that is shared by the two alternative conformations. If and how other cellular factors, for example helicases, influence early co-transcriptional events remains to be examined (Kortmann, J. and Narberhaus, F. 2012). The *N. meningitidis* homologue for cspA is NMB0838 and its transcript resulted to be strongly upregulated at 32°C. NMB1368 is a RNA helicase H belonging to the DEAD-box ATP dependent RNA helicases, another family of genes involved in post-transcriptional changes. We assessed if the deletion of NMB0838 or NMB1368 genes would affect the regulation in response to temperature downshift. Mutant strains did not show growth or vitality defects, however we excluded a direct role of both genes in NHBA thermoregulation, even if slightly decrease in NHBA expression was observed in strain grown at 30°C. Nevertheless, we showed that *nhba* RNA half-life is directly affected by temperature. *nhba* transcript is highly unstable at 37°C, but is stabilized following a temperature downshift to 30°C.

We hypothesize that low temperature triggers the formation of a secondary structure that positively affects the mRNA translatability and somehow makes it less susceptible to degradation than the structure at 37°C. We believe that the upstream regulatory region is necessary for the formation of these mutually exclusive structures, but it seems to act in concert with other regulatory elements. Part of the coding sequence is requested too, indicating that the thermosensory region extends far into the *nhba* coding region. Although we cannot exclude a possible role of small regulatory binding proteins, which would act in promoting or counteracting the alternative structures formation, we have no evidence supporting this hypothesis yet. Therefore, we tend to believe that a cis-acting element within the mRNA molecule and/or a trans-acting sRNA might be involved in the post-transcriptional regulation of *nhba* mRNA. However, a more detailed characterization would be needed to precisely determine the extent of the region and the mechanism involved.

Furthermore, we found that NHBA protein is subjected to specific turnover directly correlated to temperature changes. At 37°C the protein turnover is very rapid, but following temperature downshift to 30°C, the NHBA full length protein showed incredibly higher stability. This could also account for the discrepancy between RNA and protein expression level found in bacteria grown at 30°C until stationary phase. However, we did not investigate what and how is responsible for the differential turnover in response to temperature. Taken together, our results indicate that the overall regulation of NHBA is a cumulative effect. Multiple molecular mechanisms, such as RNA stability/translatability and protein turnover, act in concert to obtain the phenotypic differences observed.

Preliminary results obtained by comparing RNA microarray data and proteomic analysis on *N. meningitidis* strain grown at 30°C and 37°C (data not shown) led us to the observation that the effects in response to low temperature are barely detectable at RNA level, whereas a more distinct pattern is recognizable at protein level. Indeed, temperature downshift promotes not only NHBA higher surface exposure, but also a broadly enrichment in outer membrane proteins composition, such as different adhesins, siderophore receptor, soluble lytic murein transglycosylase, zinc-manganese transport system. This led us to hypothesize the existence of a global regulatory network, based on post-transcriptional/post-translational mechanisms. In the light of the new findings here reported about NHBA regulation, more in depth studies would be needed to address the hypothesis of a global regulatory network that might be used by *N. meningitidis* to fine-tune its surface composition during nasopharynx colonization.

Furthermore, NHBA is one of the three major components of the 4CMenB vaccine against serogroup B meningococcus and is a protective antigen that is able to elicit a robust immune response. Although NHBA is present in all *Neisseria* species, we have shown that its expression is variable among strains and moreover is affected by different factors such as like growth phase and temperature changes. It is therefore paramount to understand how different NHBA expression levels, either through strain variation or triggered by different growth conditions affect the bacterium's ability to resist immune response. The assay that represents the correlate of protection for meningococcal vaccines is the Serum Bactericidal Assay (SBA) and is expected to be performed at 37°C. Using an IPTG-inducible test strain as a model it was possible to

specifically estimate the influence of different NHBA expression levels on bacterial killing during the rSBA without introducing pleotropic alterations caused by different incubation temperatures. The correlation of NHBA expression and killing susceptibility has an important implication: the higher expression of NHBA during the initial steps of transmission/colonization might reflect also higher susceptibility to anti-NHBA antibodies in the nasopharynx niche. In the perspective of a broadly protective vaccine, NHBA resulted to be an important antigen in terms of cross-protection, being expressed among all *Neisseria* species. Furthermore, being highly expressed within the nasopharynx during the transmission/colonization steps, NHBA could contribute to a decrease in strain carriage and consequently to herd immunity. Recently anti-NHBA antibodies have been shown to inhibit adhesion to bronchial epithelial cells (Vacca, I. et al. 2016). Therefore, vaccine induced anti-NHBA response may be multifunctional, both bactericidal towards NHBA expressing strains, and inhibitory towards adhesion and colonization. This is the holy grail for a meningococcal vaccine. While only a minority of individuals develop the disease, the rate of carriage is considerably high. Therefore meningococcal vaccine strategies should aim to counteract invasive disease but also to reduce carriage

In susceptible non vaccinated individuals, meningococcus can occasionally cross the epithelial barrier and enter the bloodstream. During the development of invasive disease, *N. meningitidis* passes from the lower temperatures in the upper airway to the core body temperature of 37°C or higher with a febrile response to infection (Cabanac M. 1990). Additionally, within the bloodstream meningococcus has to deal with factors belonging to the complement cascade. A possible role for NHBA in serum-resistance was previously speculated, based on its ability to bind heparin (Esposito, V. et al. 2011, Serruto, D. et al. 2010). However, in light of our preliminary findings, it seems that NHBA is also involved in the initial response when the bacterium senses and adapts to the new invasive environment.

By mimicking these conditions *in vitro*, we showed that NHBA expression is also influenced by presence of serum in different ways.

Immediately after serum incubation *nhba* gene expression is induced, resulting in an increase of the NHBA protein as well. Looking at the mRNA induction within a short time-course up to 30 minutes, we observed an evolution typically attributable to a transcription induction. The initial pulse of transcription has its maximum between 5-

10 minutes and then it is stabilized to a certain level after 30 minutes of incubation (around 2-fold induction respect to T0). It has been shown that NHBA expression is not altered following 90 minutes of whole blood incubation (Echenique-Rivera, H. et al. 2011). We did not look at *nhba* RNA expression at later time points, however this is not in conflict with our observations, where the initial pulse of transcription resulted to be transient, as also suggested by protein analysis.

We also observed that simultaneously to the transcriptional induction, another mechanism takes place and affects NHBA protein expression. The full length protein is strongly processed, by a protease presents in serum generating an intense lower band around 49kDa. It has been shown that NHBA can be processed by the meningococcal protease NalP and human lactoferrin (hLf). Cleavage occurs either upstream and downstream of the NHBA Arg-rich region resulting in one of two possible cleavage fragments termed C2 and C1, respectively (Serruto, D. et al. 2010). Within the blood there is abundance of components with proteolytic activity, so it is not surprising to see an increase in C-term cleavage. However the component(s) responsible for this cleavage within the serum remain to be identified. The protein cleavage immediately starts after serum incubation, overlapping to the transcriptional induction for the first 15-30 minutes, depending on the strain analyzed. Then, the processing results to be predominant on the induced expression, which after 30 minutes is become again to the basal level and therefore leading to a decrease in the full length protein amount. What is responsible for the transcriptional response has not been identified yet, however, it seems to be something conserved among different sources of complement, since incubation with humans serum, mouse serum or baby rabbit complement leads to similar results. Despite we did not observe a direct dose-dependence response in *nhba* transcriptional upregulation, increasing the percentage of serum determines a proportional increase in NHBA cleavage, which results more evident in the MC58 strain.

Its high susceptibility to cleavage by bacterial and host proteases might let doubt about its potential as vaccine target. Monoclonal antibodies targeting the N-term of the protein are able to induce in vitro complement bactericidal killing. Functional cooperativity between antibodies targeting both the N-terminus and C-terminus of NHBA significantly contributes to extend protection against closely related phylogenetically MenB strains (Ndoni E, L.S., S Rossi Paccani, D Donnarumma, I

Bertoldi, E Bartolini, B Galli, M Bruttini, C Facciotti, P Lo Surdo, N Norais, MM Giuliani, V Masignani 2016). Our results confirm the higher functionality of an immune response directed towards the full length protein. However, by generating an uncleavable strain, we showed that the cleavage does not have a real impact on the killing susceptibility. By using an anti-NHBA mouse serum specifically directed against to the C terminal portion of the protein, we did not observe a functional response. Only minor changes are reported for the uncleavable strain grown at 30°C, when incubated with this serum.

Our results show snapshots of an extremely dynamic equilibrium in which new protein production and processing are ongoing. Furthermore, the *in vitro* correlate of protection SBA is a useful but limited tool. The set conditions, such the time of incubation, do not really represent the dynamics of the physiological killing process. Mathematical modeling of complement cascade activation dynamics highlights that after a lag phase of around 10 minutes, the reaction of C3 deposition proceeds exponentially until 25-30 minutes of incubation (Korotaevskiy, A.A. et al. 2009, Zewde, N. et al. 2016). This is in accordance with our findings about killing susceptibility triggered by anti-NHBA sera. The dynamic equilibrium between new protein production and processing turns in favor of the full length protein abundance during the first 15 minutes of serum incubation, and this time window seems to be sufficient to activate the complement cascade and therefore to trigger bacterial killing.

While antibodies directed against the C-terminus seem to need a cooperation with those directed against the N-terminal part to generate a greater functional response, the C-terminal region of the protein is very immunogenic in terms of the amount of antibodies able to recognize and bind it (Ndoni E, L.S., S Rossi Paccani, D Donnarumma, I Bertoldi, E Bartolini, B Galli, M Bruttini, C Facciotti, P Lo Surdo, N Norais, MM Giuliani, V Masignani 2016). Therefore, the susceptibility to cleavage might belong to a bacterial strategy of immune evasion by displacing antibodies that would cooperate and render the killing process even more efficient. On the other hand, the C2 fragment, released upon NalP proteolysis, has been shown to alter endothelial permeability, contributing to extensive vascular leakage (Casellato, A. et al. 2014).

Taken together, our results underline the importance of NHBA both as virulence factor and as vaccine antigen, shading light into the molecular mechanisms that regulate its expression during meningococcal colonization and invasion.

Collectively, the various gene expression strategies employed by *N. meningitidis* constitute a complex symphony of regulatory mechanisms which enable the bacterium to enter the human host, colonize it and escape its defense systems. Consequently, the orchestration of these expression strategies must ensure that the correct genes are expressed at the appropriate time and in the appropriate locale. Ultimately, understanding how these differing transcriptional and posttranscriptional regulatory mechanisms function and interact will be invaluable as these various strategies underpin the pathogenesis of *N. meningitidis*.

Chapter 2

Comparing different antigen delivery systems by using NadA as a model antigen

Immune response and vaccine design

Vaccine-induced immune effectors are essentially antibodies—produced by B lymphocytes—and capable of binding specifically to a toxin or a pathogen (Cooper, N.R. et al. 1984). The functionality of the antibodies is determined by antigen specificity, avidity and antibody subclass. This determines the ability of antisera to kill bacteria directly, but such antibodies may also neutralize toxins in the periphery, they may reduce binding or adhesion to susceptible cells/receptors and thus prevent colonization if present at sufficiently high titers on mucosal surfaces (Zhang, Q. et al. 2004). The quality of such antibody response, in terms of avidity, has been identified as a determining factor of efficacy. In addition, long-term protection requires the generation of immune memory cells capable of rapid and effective reactivation upon subsequent microbial exposure. However, the predominant role of B cells in the efficacy of current vaccines should not shadow the importance of T cell responses: T cells are essential to the induction of high-affinity antibodies and immune memory (CA, S. 2013). Antigen binding to naïve B cells initiates their activation and triggers their maturation towards the outer T cell zone of secondary lymphoid tissues. Interactions among activated B, T and dendritic cells rapidly drives B cell differentiation into Ig secreting plasma cells (MacLennan, I.C. et al. 2003). Immunoglobulin class-switch recombination from IgM towards IgG, IgA or IgE occurs during this differentiation of B cells. Both CD4+ Th1 and Th2 cells exert essential helper functions during the extrafollicular differentiation pathway, and the engagement of their CD40L molecules with CD40 on B cells may skew class-switch recombination into particular Ig classes and subclasses. In rodents, IFN-γ producing Th1 T cells promote a switch towards IgG2a, whereas Th2 cells essentially support the generation of IgG1 and IgE (via IL-4) and IgG2b and IgG3 (via TGF-β) (Deenick, E.K. et al. 2005). The situation is less clear-cut in humans, where IgG1 antibodies frequently predominate regardless of the polarization of T cell help. In experimental animal models, numerous factors influence the preferential differentiation of CD4+ T cells towards the Th1 or Th2 pathways (Swain, S.L. 1995). These determinants include the dose of antigen, lower vaccine doses being classically associated with preferential Th1 responses, and the route of administration, which targets distinct populations of dendritic cells. However, the main determinant of CD4+ T cell differentiation is the extent and type of dendritic cells activation by the innate system (Kapsenberg, M.L.

2003). Meningococcal glycoconjugate vaccine against four of the five disease-associated serogroups A, C, W-135 and Y (Gasparini, R. et al. 2011), is an example of overcoming the limitations of the related polysaccharide vaccine against serogroup C, which acts in a T-independent manner, therefore resulting in lack of immune memory generation. This approach is not suitable for vaccine design against *N. meningitidis* serogroup B because of structural similarity of its capsular polysaccharide to the sialic acid found in human neural tissue (Finne, J. et al. 1987, Finne, J. et al. 1983). Therefore, novel strategies, based on protein vaccines, have been developed to overcome this hurdle. The use of chemically detoxified OMV-based vaccines (dOMV) against MenB has been explored since the 1970s and public health interventions in countries such as Cuba, Norway, and New Zealand (Sierra, G.V. et al. 1991); (Oster, P. et al. 2005) have proven the concept of their efficacy. However, a significant limitation of these vaccines is the breadth of coverage provided. Minor non-porin proteins in the outer membrane are responsible for the cross-protection and different strategies, such as the 4CMenB vaccine, use these minor proteins to construct a more universal vaccine. 4CMen has demonstrated its safety (Beeretz I., M.S., A. Finn, P. Heath, A. Collinson, G. Bona, S. Esposito, P. Dull, E.Ypma, D. Toneatto, A. Kimura, C. Oeser, M. West, T. John, A. J. Pollard, and t. E. M. B. V. S. Group 2011, Esposito S., T.V., A. Kimura, E. Ypma, D. Toneatto, and P. Dull 2010) and its efficacy in inducing a protective immune response in all age groups against the majority of MenB strains (Findlow, J. et al. 2010, Snape, M.D. et al. 2010). Within 4CMenB formulation dOMV are combined with three major recombinant antigens. The recombinant antigens induce immune responses to a high number of serogroup B strains and the OMV potentiate the immune response to them (Acevedo, R. et al. 2014). Novel strategies are envisaged to obtain OMV from recombinant *N. meningitidis* strains, where LPS has been genetically detoxified (lpxL1-mutants), avoiding the need for detergent extraction (van de Waterbeemd, B. et al. 2010). In fact, *lpxL1* deletion results in a strain with penta-acylated LOS, which has reduced endotoxin activity in relation to the wild-type hexa-acylated LOS strain. Furthermore, mutant strains with over-expressed protein vaccine antigens, like PorA and fHbp, naturally inserted into the membranes have been constructed (Weynants, V. et al. 2009) (Koeberling, O. et al. 2011).

The Neisserial adhesin A (NadA)

The Neisserial adhesin A (NadA) is a trimeric autotransporter protein of *N. meningitidis* belonging to the group of oligomeric coiled-coil adhesins. It is formed by three identical inter-winded polypeptides organized with a variable N-terminal globular portion, called the passenger, protruding outside and comprising the binding site(s) for target cell receptors and a conserved C-terminal domain barrel-shaped, that drives the self-secretion and anchors the protein onto the bacterial outer membrane. These two domains are separated by a flexible coiled-coil stalk comprising a leucine zipper, which have a propensity to form trimers. (Hoiczyk, E. et al. 2000, Malito, E. et al. 2014, Tavano, R. et al. 2011).

Through the passenger domain NadA mediates adhesion to human epithelial cells (Capecchi, B. et al. 2005), assuming an important role during meningococcal colonization of the human upper respiratory tract. However, it is involved in all steps of meningococcal physiopathology. In fact, NadA not only promotes invasion of human epithelial cells (Capecchi, B. et al. 2005), but it also exerts an immune-modulatory action on myeloid cells by binding macrophages and dendritic cells. This leads to the maturation of dendritic cells as well as the activation and differentiation of monocytes into macrophages (Franzoso, S. et al. 2008, Mazzon, C. et al. 2007). Furthermore, interacting with the human endothelial receptor LOX-1 it is hypothesized to promote the crossing of the blood-brain barrier (Scietti, L. et al. 2016).

The *nadA* gene is an independent genetic unit (Comanducci, M. et al. 2002) and is the result of an insertion of foreign DNA in the meningococcal genome most likely through horizontal gene transfer and subsequent limited evolution to generate five variants, each of them including a number of subvariants (Comanducci, M. et al. 2002 2009, Comanducci, M. et al. 2004).

NadA is a risk factor for the development of meningococcal disease, being present in 50% of the disease-associated strains and overrepresented, almost 100%, in hypervirulent meningococcal lineages (clonal complexes ST-32, ST-8, ST-11, ST-1157 and ST-213) (Bambini S., F.J., Klaus H., Taha M.K., Stefanelli P., Caugant D.A., Lucidarme J., Gilchrist S., Borrow R., Vogel U. et al. 2011, Comanducci, M. et al. 2004, Wang, X. et al. 2011). The *nadA* gene is carried by about 30% of pathogenic isolates collected from patients in 5 European countries and the US (Bambini S., F.J., Klaus H., Taha M.K., Stefanelli P., Caugant D.A., Lucidarme J., Gilchrist S., Borrow R., Vogel U.

et al. 2011, Wang, X. et al. 2011), whereas only 5% of carriage isolates obtained from healthy individuals harbour the gene (Comanducci, M. et al. 2004). Furthermore, *nadA* is absent from *N. gonorrhoeae* and the commensal *N.lactamica* and *N. cinerea* isolates.

The *nadA* gene shows growth phase dependent expression, reaching a maximal level in the stationary phase (Comanducci, M. et al. 2002, Metruccio, M.M. et al. 2009, Seib, K.L. et al. 2010). The expression of *nadA* is also subjected to phase variation, through the presence of a variable length tetranucleotide repeat upstream of its promoter (Martin, P. et al. 2003, Metruccio, M.M. et al. 2009). For this reason *nadA* expression varies both between different strains, as well as within a single strain, having variants where changes in the repeats number result in promoters with low, medium or high activity (Martin, P. et al. 2003, Metruccio, M.M. et al. 2009). However, the major mediator of the phase variable expression of *nadA* is NadR, a MarR-like protein which binds with high affinity to two sequences flanking the variable repeat region, acting as repressor through sterically hindering RNA polymerase to access to the promoter. Changes in the number of repeats affect the ability of NadR to repress the *nadA* promoter (Metruccio, M.M. et al. 2009). As typical for MarR-like proteins, a small molecule ligand, 4-hydroxyphenylacetic acid (4HPA), was identified which is able to relieve the DNA binding activity of NadR and derepress/induce *nadA* expression (Metruccio, M.M. et al. 2009). 4HPA is a catabolite of aromatic amino acids and is secreted in human saliva (Takahama, U. et al. 2002). This metabolite may act as a relevant niche signal to meningococci present in the oropharynx, which is bathed in saliva, for the modulation of the activity of NadR.

The proposed model (Figure 2.8) shows that NadR can bind to and repress *nadA* through a looping mechanism, which may result in steric hindrance of RNA polymerase access to the promoter, possibly through dimer-dimer interactions on multiple binding sites. The interaction of NadR dimers bound at different operators might be facilitated by the interplay of the Integration Host Factor (IHF), a histone-like protein which was demonstrated to bend DNA upon binding (Swinger, K.K. et al. 2004). Following the binding of 4HPA or other biologically relevant signals (Figure 8, lower panel), the NadR protein can be stabilized in a conformational state which is not able to efficiently bind the *nadA* promoter region, thus causing the induction of downstream gene.

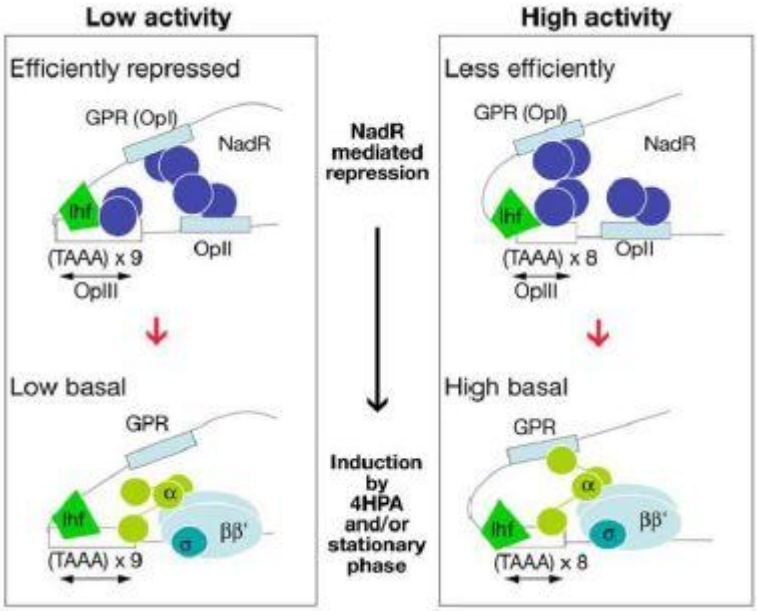


Figure 2.8 Model of the *nadA* promoter regulation.
Two promoter variants with 9 and 8 repeats representing low activity and high activity promoter phase variants, respectively, highlighting the ability of NadR to efficiently or less efficiently repress the promoters (top panels) and NadR-independent effects on the derepressed promoter basal levels possibly due to differential contacts with the α-subunit of RNAP (bottom panels) due to different spatial organization of the NadR and RNA polymerase contact points resulting from the different number of repeats. (Metruccio, M.M. et al. 2009).

NadA is characterized by a high degree of biochemical stability to heat, detergent and reduction (Magagnoli, C. et al. 2009). NadA is capable of inducing strong cellular immune responses (Bowe, F. et al. 2004), bactericidal antibodies in animal models (Bowe, F. et al. 2004, Comanducci, M. et al. 2002, Giuliani, M.M. et al. 2006, Pizza, M. et al. 2000) and in humans (Litt, D.J. et al. 2004) as well as the secretion of pro-inflammatory signals from monocytes and macrophages (Franzoso, S. et al. 2008). Therefore it can be used as a model antigen to investigate how different delivery systems stimulate the host immune response and helping in next generation vaccines design.

5. Results

5.1. NadA overexpression on MenB and *E.coli* nOMV

In order to produce native outer membrane vesicles (nOMV) carrying high quantities of NadA, we overexpressed NadA in MenB and *E.coli* systems and prepared OMV from these strains. Two overexpressing strategies were used for homologous or heterologous expression. It has been shown that despite its variable expression levels among strains, NadA can be overexpressed in all strains where the gene is present, following the removal of its regulator NadR (Fagnocchi, L. et al. 2013). The level of expression is critical for the breath of coverage (Fagnocchi, L. et al. 2013). 5/99 is one of the higher NadA expressing strains among meningococcal B strains (Metruccio MM, 2009) and its potential to overexpress the *nadA* gene is therefore one of the highest. We selected this strain to generate nOMV overexpressing NadA following the deletion of the *nadR* gene by replacing it with an antibiotic resistance cassette. Details of the strain used are listed in Material and methods section. For heterologous overexpression, the BL21Δ*tolR E.coli* strain was transformed with a plasmid carrying the *nadA* variant 3 gene under the control of an IPTG-inducible promoter and used to prepare nOMV from the heterologous *E.coli* system. A 5/99 strain, knocked out for *nadA*, was used to produce nOMV void of NadA as negative control. nOMV were prepared and purified as indicated in Material and Methods section, and their physical properties were analyzed using immunogold electron microscopy (Figure 5.1), which confirmed the presence of NadA on their external surface. The vesicles appeared round, intact and with a size distribution ranging from 20 to 100 nm in diameter. In order to characterize the proteomic content of nOMV produced by the recombinant over-expressing strains, total protein extracts from 0.5 or 1 μg of nOMV were compared to different quantities of the recombinant protein, used as titration curve. Proteins were separated on SDS-PAGE gels and either visualized by Simply Blue™Safe staining or blotted onto nitrocellulose and the protein bands of interest detected using polyclonal mouse sera (Figure 5.1 B and C, respectively). nOMV from *E.coli* and MenB strains both showed a band at the NadA expected molecular weight in its trimeric form, visible in the SDS-PAGE (Figure 5.1 B). The NadA expression was confirmed by Western blot analysis. Immunoblotting revealed the presence of some bands in *E.coli* OMV at a lower molecular weight, compatible with the monomeric form of the protein. Furthermore,

the NadA overexpression in the meningococcal recombinant strain respect to the wild type strain became evident (Figure 5.1 C). Densitometry analysis performed after immunoblotting and infrared detection allow us to estimate that the quantity of NadA expressed on the nOMV correspond to 20-25% of the total protein content (Figure 5.1 C).

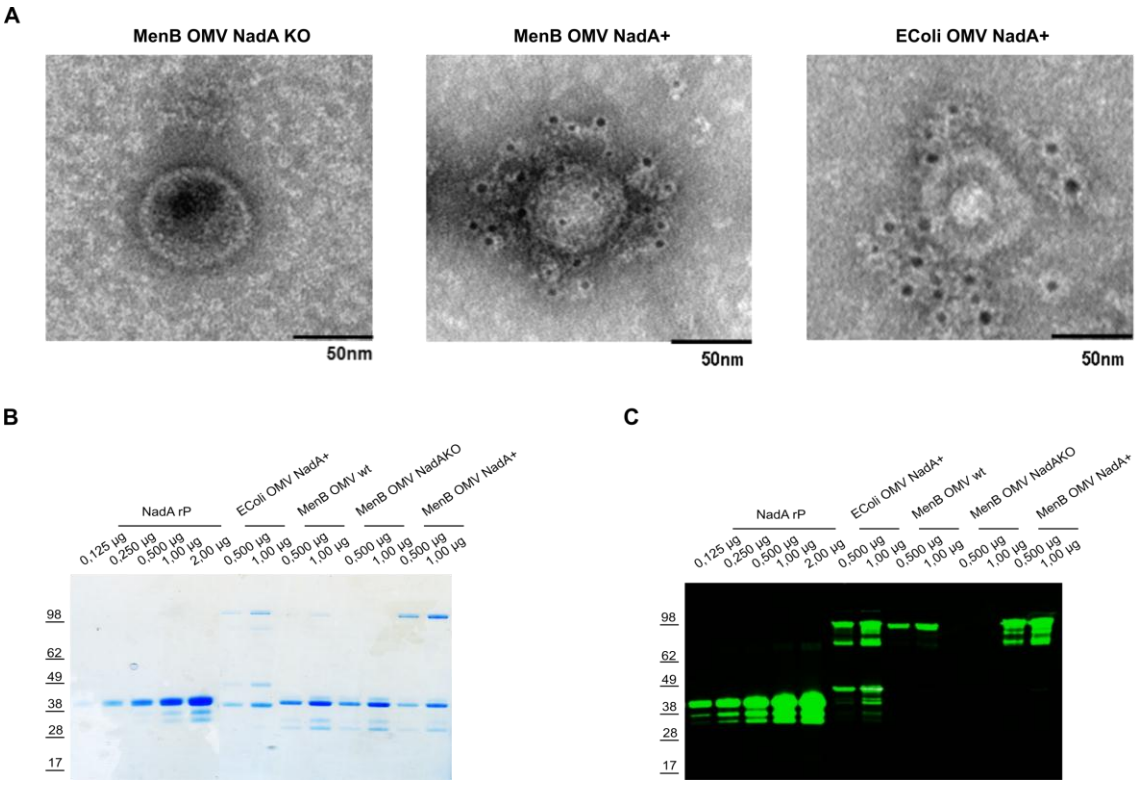
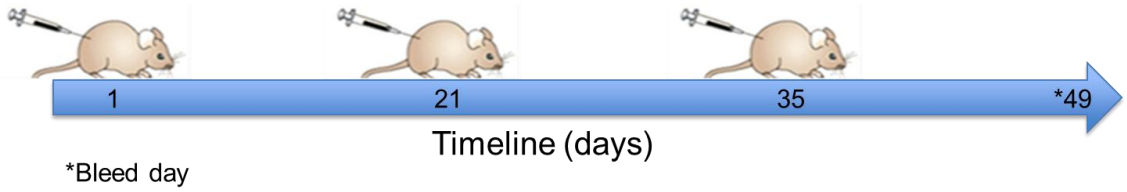


Figure 5.1 Homologous and heterologous expression of NadA on nOMV. (A) Immunogold electron microscopy on nOMV produced by meningococcal 5/99 ΔlpxL1 ΔNadA and 5/99 ΔlpxL1 ΔNadR strains and *E.coli* BL21ΔTolR_pET21b_NadA strain. Total protein extracts from nOMV were performed and separated by SDS-PAGE, using the recombinant protein (NadA rP) to set a standard curve. **(B)** Simply Blue™Safe staining and **(C)** Western blotting.

5.2. Prototype nOMV vaccines elicited high titers of α-NadA antibodies

CD-1 mice were immunized intra-peritoneally three times with 5 µg of each nOMV or 20 µg of recombinant form NadA, formulated with aluminum hydroxide to evaluate the ability of each vaccine formulation to induce broadly protective α-NadA antibodies (Figure 5.2). nOMV prepared from meningococcal NadA knocked-out strain were used as a negative internal control.



Antigen	Dose	Adjuvant	Route
NadA rP	20 µg	Alum 3mg/ml	IP
MenB OMV NadA KO	5 µg	Alum 3mg/ml	IP
MenB OMV NadA+	5 µg	Alum 3mg/ml	IP
EColi OMV NadA+	5 µg	Alum 3mg/ml	IP

Figure 5.2 Immunization scheme. Upper panel: five CD-1 mice were immunized intra-peritoneally (IP) three times, as indicated. Bottom panel: composition of different vaccines formulations**.**

The antibody titers elicited were evaluated by ELISA analysis using post-III immunized sera of individual mice (Figure 5.3). All antigen groups elicited antibody titers which were significantly higher respect to the negative control, MenB OMV NadA KO.

MenB OMV NadA+ showed slightly lower but less variable response among individual mice. However, no significative differences are reported among the three groups in terms of geometric mean titres elicited.

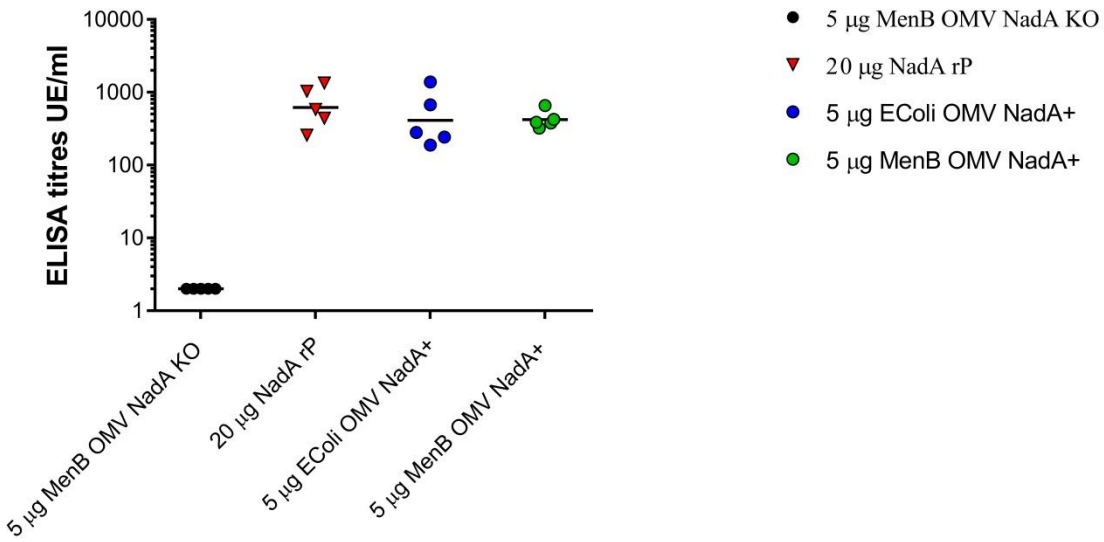


Figure 5.3 Analysis of sera from mice immunized with nOMV or with recombinant NadA protein. IgG α-NadA antibody titers elicited measured by ELISA. Each dot represents an individual mouse serum while the line indicates the median value within each immunization group.

5.3. Evaluation of the bactericidal activity of the antibodies elicited through SBA assay

To estimate the bactericidal activity of the elicited antibodies, mice sera were tested against BZ83 meningococcal natural strain. This strain was selected as test strain being mismatched for PorA variant, usually the immunodominant antigen of OMV, which elicits a PorA specific and not cross response.

As expected accordingly to ELISA titres, no killing was achieved with the serum derived from mice immunized with MenB OMV NadA KO negative control. Instead, a great functional response was achieved with sera derived from mice immunized with *E.coli* OMV NadA+ and MenB OMV NadA+ suggesting all bactericidal activity measured on the BZ83 test strain is NadA-mediated. The functionality of the antibody elicited by OMV formulations is much greater than the ones elicited by the recombinant protein (Figure 5.4), indicating that even at lower dose of the antigen, the different vaccine formulation improves the functional immune response.

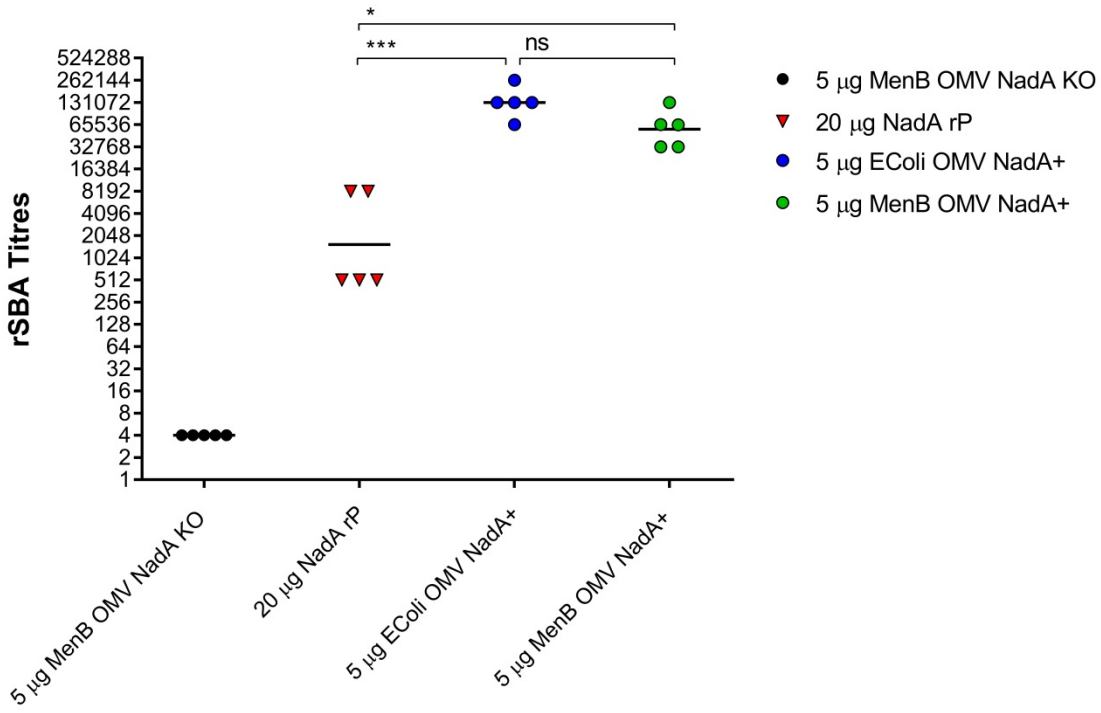


Figure 5.4 Serum bactericidal antibody (SBA) titers against BZ83 meningococcal strain. Dots represent SBA titers of individual mouse sera against the defined strain. Statistical analysis was performed using Kruskal-Wallis multiple comparisons test (ns: not significant; *p<0.05; ***p<0,001).

Subsequently, IgG subclass (IgG1, IgG2a, IgG2b and IgG3) levels were determined (Figure 5.5 A).

All the vaccine formulations generated antibodies of all IgG subclasses (IgG1, IgG2a, IgG2b and IgG3). However, despite the total IgG measured by ELISA resulted to be comparable among all the vaccine formulations, immunization with the recombinant protein elicited predominantly IgG1. On the other hand, *E.coli* and MenB OMV NadA+ stimulated a significant increase in IgG2a, IgG2b and IgG3 production. It has been shown that different mouse IgG subclasses exert different efficiency in functional assays (Michaelsen et al 2003), where IgG3 and IgG2b resulted to be the most efficient isotypes in SBA performed against meningococcal strains (IgG3>>IgG2b>IgG2a>>IgG1). Therefore, the predominance of such IgG subclasses in OMV vaccine formulations, may explain the differences observed in the functional assay in comparison to the recombinant protein formulation.

Furthermore, the IgG2/IgG1 ratio was calculated for all the sera tested, highlighting that the magnitude of Th1 response seems to be enhanced in OMV based vaccine formulations (Figure 5.5 B).

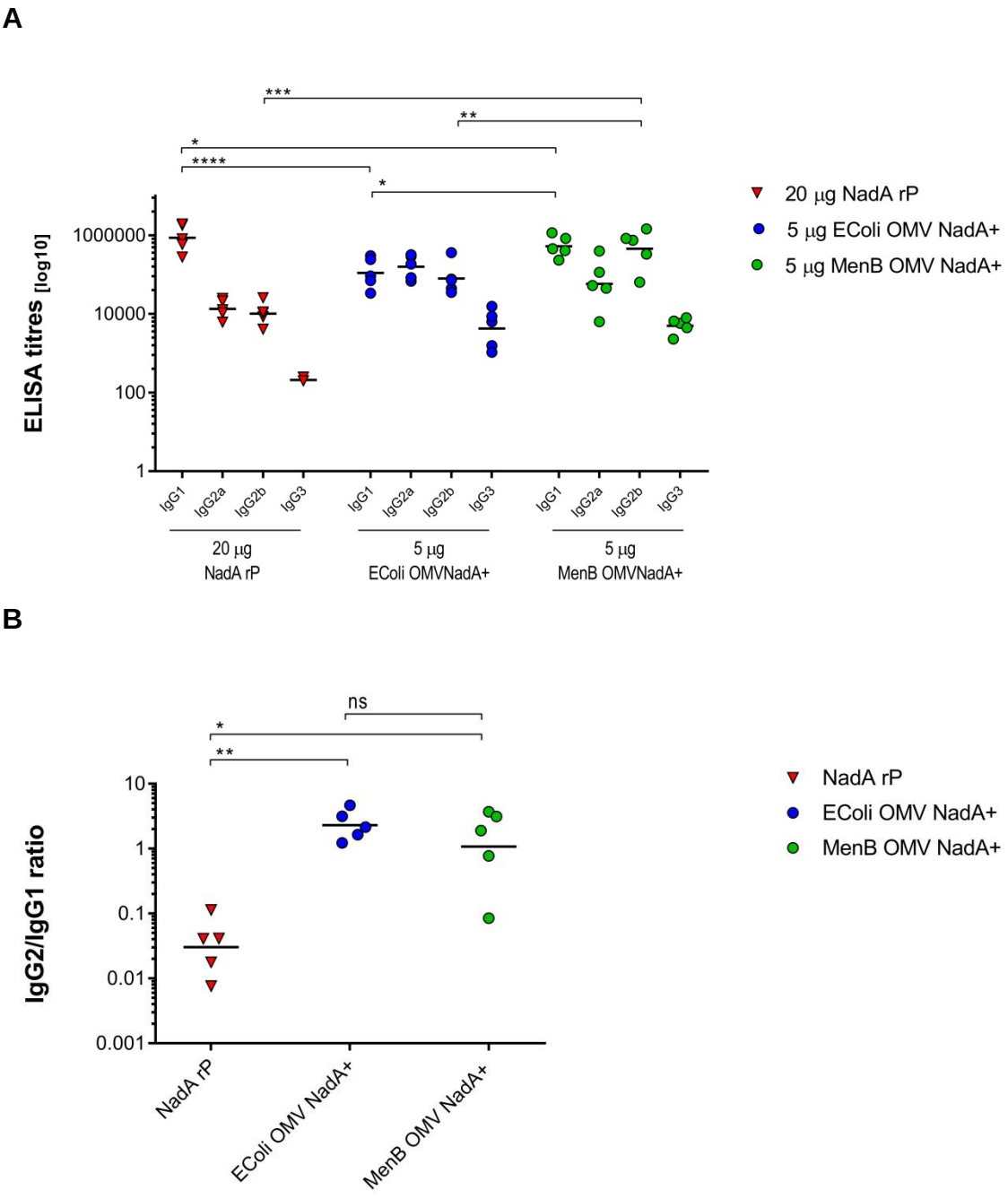


Figure 5.5 IgG subclasses elicited by different vaccine formulations.(A-B) The ratio IgG2/IgG1 (lower panel) was calculated considering the sum of both IgG2a and IgG2b. Statistical analysis was performed using Two-way ANOVA followed by uncorrected Fisher's LSD multiple comparison test (upper) or Kruskal-Wallis multiple comparison test (lower) (**** $p<0.0001$; *** $p<0.001$; ** $p<0.01$; * $p<0.05$; ns : not significant).

5.4. Inhibition of *E.coli*-NadA var3 adhesion to Chang epithelial cells with different sera

It was previously shown that expression of NadA on the surface of *E.coli* (*E.coli*-NadA) promotes bacterial adhesion to Chang epithelial cells (Capecchi, B. et al. 2005). Particularly, the N-terminal portion was found to be crucial for the interaction. In order to investigate the ability of the above mentioned sera to interfere in the first step of bacteria pathogenesis, we decided to pre-incubate *E. coli*-NadA with the selected sera before proceeding with the infection scheme on Chang cell monolayers (Figure 5.6). Readout of the infection was provided by the number of cell-associated colony-forming units (cfu) from supernatants of cell lysates. Results of these experiments are shown in Figure 5.6. The number of cfu obtained in absence of sera (*E.coli*-NadA) was assigned to be 100% of adhesion while the percentage of cfu obtained infecting cells with *E.coli*-peT indicates the maximum level of inhibition. These two values were taken as references to calculate the magnitude of adhesion after pre-treating with the selected sera. Sera raised against nOMV from a MenB strain KO for NadA (α-OMV MenB KO NadA), as expected, do not interfere with bacteria adhesion and resulted in 100% adhesion. For all the others vaccine formulation there is an inhibitory effect on bacterial adhesion at both sera dilutions, with the highest effect elicited by the lowest dilution, as expected. Maximum levels of inhibition were obtained with sera raised against OMV carrying NadA (α-OMV EColi $NadA^+$ and α-OMV MenB $NadA^+$) regardless of the bacteria used to generate OMV.

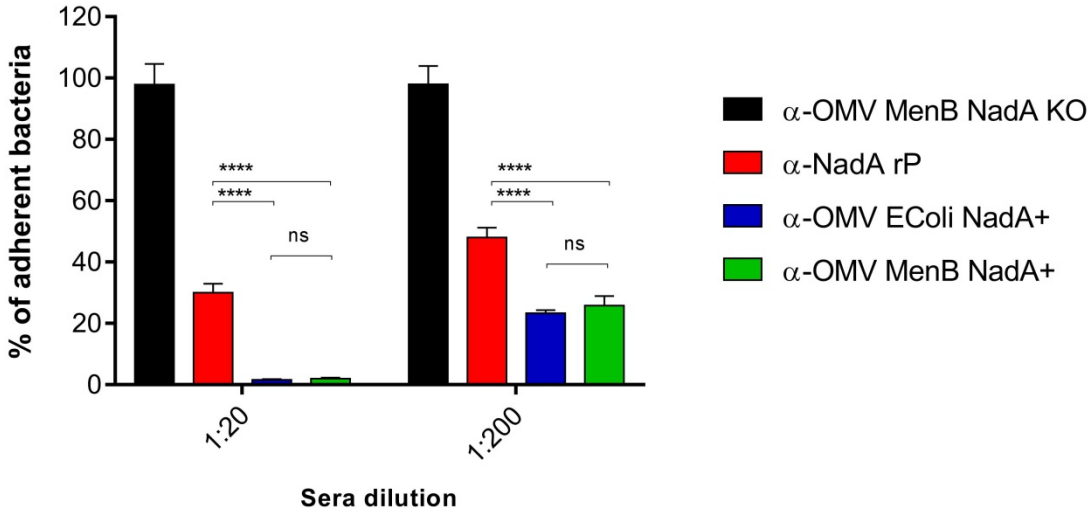


Figure 5.6 Inhibition of NadA-mediated adhesion. Statistical analysis was performed using Two-way ANOVA followed by uncorrected Fisher's LSD multiple comparison (**** $p<0.0001$; ns: not significant).

6. Discussion and conclusions

The four component recombinant protein vaccine 4CMenB has demonstrated its safety (Beeretz I., M.S., A. Finn, P. Heath, A. Collinson, G. Bona, S. Esposito, P. Dull, E.Ypma, D. Toneatto, A. Kimura, C. Oeser, M. West, T. John, A. J. Pollard, and t. E. M. B. V. S. Group 2011) (Esposito S., T.V., A. Kimura, E. Ypma, D. Toneatto, and P. Dull 2010) and its efficacy in inducing a protective immune response in all age groups against the majority of MenB strains (Findlow, J. et al. 2010) (Snape, M.D. et al. 2010). The development of next-generation vaccines requires the combination of diverse strategies, such as different delivery systems/adjuvants, to present the antigen in a manner that can elicit an adequate and efficient immune response.

In this work we compared the immune response elicited by recombinant protein and nOMV delivery systems, using NadA as model antigen.

Outer membrane vesicles are very complex supramolecular structures. They contain immune stimulators (e.g., LPS, proteins, and DNA) and antigenic molecules that can be delivered to immune competent cells of the immune system to trigger maturation as well as activation signals. Therefore, OMV have an intrinsic adjuvant effect over loaded antigens from bacteria, but also over heterologous antigens that can be incorporated or combined in a single formulation.

Since nOMV generally have high levels of endotoxin activity due to the presence of lipooligosaccharide (LOS), the strains used for MenB nOMV-vaccine preparation was genetically engineered to reduce/abolish their reactogenicity. The innate human immune system senses microbes through a number of receptors that can recognize a variety of microbial structure (Manicassamy, S. et al. 2009). LOS, a major component of Gram-negative bacteria, is recognized by TLR4 and MD-2 by host innate immune cells leading to the production of pro-inflammatory cytokines that initiate and shape the adaptive immune response. Lipid A that anchors LOS in the outer membrane of the bacterium is primarily responsible for LOS endotoxin activity (Maeshima, N. et al. 2013, van der Ley, P. et al. 2001). In particular, the specific acylation pattern of the lipid A moiety largely determines its biological activity. In order to improve the vaccine's biosafety we directed the integration of an antibiotic resistance cassette into the lpxL1 gene, thereby inactivating it. The lpxL1 gene deletion alters the chemical composition of the lipid A leading to penta- instead hexa-acetylated molecules (Fisseha, M. et al.

2005, van der Ley, P. et al. 2001). Even though certain strains of *Neisseria* can live without LOS (Steeghs, L. et al. 1998), we decided to not remove the capacity of the bacterium to produce LOS as it has adjuvant properties, stimulating the antibody response against outer membrane proteins (OMPs) (van der Ley, P. et al. 2001). In particular, the lpxL1 LOS retains adjuvanticity similar to wild-type meningococcal LOS while reducing its toxicity as measured by a TNF-alpha induction assay (van der Ley, P. et al. 2001).

We were able to obtain MenB OMV overexpressing NadA by knocking out the *nadR* gene (Fagnocchi, L. et al. 2013), and *E.coli* OMV overexpressing NadA by using an expression plasmid carrying the *nadA* gene. Both the homologous and the heterologous bacterial expression systems showed high level of NadA expressed on the surface. Surprisingly, in MenB and *E.coli* OMV, NadA represents the 20-25% of the total protein content within the outer membrane composition.

20 μg of NadA v3 recombinant protein or 5 μg of OMV-based vaccines were used to immunize mice and evaluate the ability to induce bactericidal antibodies. Nevertheless, all vaccine formulations containing NadA were able to induce α-NadA antibodies at comparable levels. This suggests that the natively expressed NadA in nOMV are significantly more immunogenic that the recombinant protein, and/or that the nOMV *per se* have an adjuvant property.

Moreover, the quality of the antibodies elicited by OMV vaccine formulations was superior to that of the antibodies elicited by the recombinant proteins. Even though the α-NadA antibody titers were comparable, the antibodies elicited by the vesicles were able to elicit a more potent functional response. Remarkably, sera obtained immunizing with MenB OMV that do not express NadA, do not show any killing activity on BZ83 strain. This observation underlines the specificity and the safety in eliciting OMV-driven immune response.

There could be multiple reasons for higher functional immune responses elicited by an antigen delivered on an OMV.

(I) NadA is characterized by a high degree of biochemical stability to heat, detergent and reduction (Magagnoli, C. et al. 2009). Even though, when expressed on OMV surface, NadA shows a great stability on its trimeric form. OMV overexpressing NadA accurately represent the bacterium surface, therefore faithfully presenting the antigen in its natural

conformation. The α-NadA antibodies elicited by the OMV vaccines may have targeted surface-exposed epitopes better than the antibodies elicited by the recombinant NadA vaccine.

(II) The high density antigenic array presented by a nanoparticle, such an OMV, provides a molecular scenario where multiple binding events occur simultaneously between the OMV and the host cell B-cell receptors, leading to BCR cross-talking and activation of B cells in a T-independent fashion which can result in an increased response (CA, S. 2013). This multivalent molecular and cellular setting favors the fruitful network of stimulatory interactions, as opposed to the weaker effect of monovalent binding afforded by single soluble recombinant antigens. Indeed, the high avidity for the OMV provided by the multivalent interaction constitutes a critical step in the induction of a potent immune reaction (Lopez-Sagaseta, J. et al. 2016).

(III) The presence of natural immune potentiators or coadministered adjuvants is known to influence the type of immune response. OMV vaccine formulations do contain LPS and bacterial DNA, which are agonists for TLR4 and TLR9, respectively (Akira, S. et al. 2004). Activation of these pathogen recognition receptors stimulates and directs the humoral immune response in a T-cell dependent or independent way (Bekeredjian-Ding, I. et al. 2009).

(IV) Finally, OMV phenotypic properties should be taken into account. The nanoparticle structures may affect the uptake by different cell types with respect to efficiency in uptake and routing within the cells or drain faster (unprocessed) to the lymph nodes (Bachmann, M.F. et al. 2010), thereby affecting the immune responses induced.

In immunization studies a complete immunoglobulin subclass profiling, especially regarding IgG2b and IgG3, is often absent (Mills, K.H. et al. 1998, Raeven, R.H. et al. 2015) but should be taken into account for a better understanding of host-pathogen interaction and moreover, for vaccine design. Here, we show that different delivery systems evoke differentially flavored immune response. We found that the differences in functionality were associated with distinct antibody subclass repertoires elicited by the different vaccine formulations. OMV were able to elicit high titers of IgG2a, IgG2b

and IgG3, besides IgG1, while, the recombinant proteins elicited high titers of mostly IgG1.

The human IgG subclasses are known to differ in their ability to activate complement mediated responses. Specifically, IgG1 and IgG3 antibodies activate complement efficiently, whereas IgG2 antibodies are effective mainly at high epitope density (Michaelsen, T.E. et al. 1991). IgG3 antibodies show the highest affinity in complement activation and the relative balance of the IgG subclasses in any immune complex helps to determine the strength of the inflammatory processes that follows (Michaelsen, T.E. et al. 1991). Alternatively, murine IgG2 exhibit the most complement activation, and the increases in the elicitation of IgG2 subclasses could explain the higher functional responses of the OMV immunizations in mouse sera. The formation of antibody-producing B-cells can occur either through a T-cell dependent or independent way. In the case of T-cell involvement, the type of cytokines secreted, e.g. IFNγ, IL-4, IL-5, and IL-17, determines the IgG subclass production by B-cells (Mitsdoerffer, M. et al. 2010, Raeven, R.H. et al. 2015, Stevens, T.L. et al. 1988). In mice, Th1 type responses promote the production of IgG2a, whereas Th2 responses stimulate the formation of IgG1 (Germann, T. et al. 1995). In addition, production of IgG2b seems to be linked to a Th17 type response (Mitsdoerffer, M. et al. 2010). Whereas all types of T cell help stimulate the secretion of IgG3 (Germann, T. et al. 1995, Stevens, T.L. et al. 1988), the highest IgG3 formation is achieved by T-cell independent antibody responses by B-cells (Quintana, F.J. et al. 2008).

Therefore, the presence of LPS and DNA in OMV vaccine formulations might partially explain the different subclass responses.

This indicates that the sum of all interactions within and between activated pathways determines the environmental conditions in which the B-cells reside, and consequently the outcome of the humoral response.

NadA mediates adhesion to human epithelial cells (Capecchi, B. et al. 2005), assuming an important role during meningococcal colonization of the human upper respiratory tract and moreover its expression is induced in vivo by signals present in the saliva (Fagnocchi, L. et al. 2013). Additional evidence of the crucial role of NadA head domain in meningococcus pathogenesis are provided by Malito and colleagues (Malito, E. et al. 2014) that mapped the epitope of the neutralizing bactericidal mAb 33E8 on this portion of the adhesin. Here, we showed that all the sera raised against

NadA are also able to interfere with adhesion. However, maximum levels of inhibition were obtained with sera raised against OMV carrying NadA, regardless of the bacteria used to generate OMV.

Overall, these data suggest that the use of OMV as a vehicle probably results in a better exposure of the antigen to the immune system. Conformational epitopes important to mediate host-pathogen interaction can be better recognized, therefore resulting in a stronger inhibition of NadA-mediate adhesion.

Taken together all these data support the potential of OMV as vaccine platform, and also the great potential of NadA as a model antigen.

7. Materials and Methods

7.1. Bacterial strains and culture conditions

All the strains used in this study are listed in Table 8.1 (Appendix section). *N. meningitidis* strains were routinely grown on chocolate agar (Biomerieaux), Gonococcus (GC) agar (Difco) supplemented with Kellogg's supplement I, or on Mueller-Hinton (MH) agar (Difco) at 30°C or 37°C (as specified for single experiments), 5% CO_2 overnight. 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. Colonies from overnight growth were used to inoculate 7 ml cultures at an optical density at 600 nm (OD_{600}) of ~0.05. The cultures were incubated at 37°C with shaking until early exponential (OD_{600} ~0.25) or mid-exponential phase (OD_{600} ~0.5) or as specified in the individual experiments. When required isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) was added to culture medium at the indicated final concentrations.
Strains were stocked in GC medium with 15% glycerol and were stored at −80°C.
Escherichia coli DH5-α (Hanahan, D. 1983), HK100 (Klock, H.E. et al. 2009) and BL21(DE3) ΔTolR (Berlanda Scorza et al., 2008) strains were grown in Lysogeny broth (LB) and when required, ampicillin, kanamycin and chloramphenicol were added to achieve final concentrations of 100, 25 and 10 μg ml^{-1} respectively.

7.2. Generation of plasmids and *N. meningitidis* new recombinant strains

All recombinant strains and plasmids used in this study are listed in Table 8.1, while all the oligonucleotides used are listed in Table 8.2 (Appendix section). DNA manipulations were carried out routinely as described for standard laboratory methods (Sambrook J, 1989). Generation of all deletion mutants (Δ*nhba*), MC58Δ*nhba*-C*nhba* and MC58Δ*nhba*-Ptac_*nhba* strains were obtained as described in (Serruto, D. et al. 2010). Generation of 5/99 recombinant strains were obtained as described in (Fagnocchi, L. et al. 2012) and (Koeberling, O. et al. 2008). To obtain the pCOM_14aa_mCherry plasmid, used to generate the MC58Δ*nhba*-Pwt_mCherry strain, polymerase incomplete primer extension (PIPE) PCR (Klock HE, 2009) was performed. The coding sequence of the mCherry gene optimized for Neisseria (Invitrogen) was amplified with mCherryFw and mCherryRev primers. The pCOM_*nhba* plasmid was

used as template to amplify the backbone with divergent primers: Vector Fw, that maps in the chloramphenicol resistance cassette, and Pwt_14aa Rev that allow the amplification of the intergenic region upstream to *nhba*, including its own promoter and the first 14 amino acids of the coding sequence. NmmCh_Nde Fw and NmmCh_Nsi Rev primers were used to amplify the mCherry sequence (Invitrogen). Afterwards, the amplicon was subcloned on pCOM-PInd vector using the restriction sites included within the sequence.

Upstream and downstream flanking regions that drive the homologous recombination to obtain NMB0838 or NMB1368 deletion, were amplified from MC58 genomic DNA through fusion PCR and then cloned into pGEM-Teasy plasmid. The chloramphenicol resistance cassette was therefore inserted by using the BamHI site placed in between of them. Site-directed mutagenesis was performed on pCOM_C*nhba* plasmid to generate mutants Mut_1-Mut_7, by using pairs of primers indicated in Table 8.2. Due to the extent of the mutation needed to obtain the MC58_ΔTSFAmRR, two round of site-directed mutagenesis were performed. The pBS_*nhba* plasmid was used as template and amplified by using mRR Fw/Rev primers to generate pBS_mRR plasmid. Then, the pBS_mRR plasmid was amplified by using ΔTSFA Fw/Rev primers to generate the pBS_ΔTSFAmRR plasmid. All PCR amplifications were performed using the KAPA Hi-FI polymerase (KAPA Biosystem), following manifacturer's instructions. The correct nucleotide sequence of each plasmid was confirmed by DNA sequencing. Prior to transformation into *N. meningitidis*, plasmids were linearized by restriction digestion using *Spe*I (New England Biolabs) following the manufacturer's instructions. For transformation of naturally competent *N. meningitidis*, five to ten freshly grown overnight colonies were re-suspended in 30 µl of GC medium and spotted onto GC plates. 1-10 µg of linearized plasmid DNA was added, allowed to dry and incubated for 5-6 h at 37°C. Transformants were then selected on GC plates containing erythromycin (5 µg ml^{-1}), kanamycin (150 µg ml^{-1}) or chloramphenicol (5 µg ml^{-1}) after overnight incubation at 37°C. Single colonies were re-streaked on selective media and genomic DNA purified by crude lysis after overnight incubation at 37°C. Single colonies were re-suspended in 100 µl of distilled water, boiled for 10 min and centrifuged in a bench top centrifuge for 5 min at 8000 × *g*. One to three microliters of the sample were used as template for PCR analysis to check the correct insertion of the resistance marker by a double homologous recombination.

7.3. Polyacrylamide gel electrophoresis and Western blotting

N. meningitidis colonies from overnight plate cultures were re-suspended in PBS to an OD_{600} of 0.5. One milliliter of the resuspension was centrifuged for 5 min at 15000 × *g* and the pellet re-suspended in 100 µl in 2X SDS-PAGE loading buffer (50 mM Tris Cl [pH 6.8], 2.5% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 50 mM dithiothreitol [DTT]) (Oriente, F. et al. 2010). For liquid culture, 1 ml of each sample was collected and the concentration normalized in 2x SDS-PAGE loading buffer respect to the relative optical density at 600 nm. Protein extracts were separated by SDS-PAGE on NuPAGE® Novex® 4-12% Bis-Tris Protein Gels in MES 1X (Life Technologies) and transferred onto nitrocellulose membrane using an iBlot Dry Blotting System (Invitrogen). Membranes were blocked for 2 h at room temperature with PBS with 0.05% (v/v) Tween 20 (Sigma) and 10% (w/v) powdered milk (Sigma) and then incubated for 60 min at room temperature with the specific primary antibodies diluted in PBS + 0.05% (v/v) Tween 20 (Sigma) and 3% (w/v) powdered milk (Sigma). A horseradish peroxidase-conjugated anti-mouse IgG antibody and the Western Lightning ECL (Perkin Elmer) were used according to the manufacturer's instructions and the densitometry quantification was performed by using the ImageJ 1.6 software.

7.4. Quantitative real-time PCR (qRT-PCR) experiments.

Bacterial cultures were grown in 7 ml of liquid medium to OD_{600} as specified for the individual experiments. Three ml of the culture were then poured onto 3 ml of frozen medium to immediately chill the culture and stop transcriptional changes. Cells were then harvested by centrifugation at 3400 × *g* for 10 min. Total RNA was isolated using an RNeasy Mini kit (Qiagen) as described by the manufacturer. A second step of DNase treatment was performed using RQ1 RNase-free DNase (Promega), for one hour at 37°C and purified with the RNeasy Mini kit. RNA was quantified using a Nanodrop 1000 spectrophotometer and its overall quality was assessed by running samples on a 1% agarose gel. 2 µg of total RNAs were reverse-transcribed using random hexamer primers and SuperScript® II RT (ThermoFisher), following

manufacturer's instructions. As negative controls, all RNA samples were also incubated without reverse transcriptase.

Quantitative real time-PCR was performed in triplicate per sample in a 25 µl reaction volumes using Platinum® SYBR® Green qPCR SuperMix-UDG with Rox (ThermoFisher) according to the manufacturer's instructions and containing 2.5 ng of cDNA, and 0.4 µM of gene-specific primers (See Table 8.2). 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 (Livak, K.J. et al. 2001). Two-way ANOVA was used to calculate statistical significance and significance levels are indicated on the respective figures (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

7.5. Flow cytometry

Strains were grown in GC or MHB broth until an OD_{600} of 0.25 or 0.5, collected by centrifugation (8000 × *g* for 5 min), incubated for 1 h at room temperature with secondary antibody alone, mouse monoclonal antibodies or mouse polyclonal sera diluted to specific concentrations indicated in the respective experiments in PBS containing 0.1% (w/v) BSA. The cells were then incubated for 1 h at room temperature with a secondary rabbit anti-mouse immunoglobulin G (whole molecule) FITC-conjugated (Sigma) and then incubated for 2 h at room temperature in PBS containing 0.5% (w/v) paraformaldehyde (PFA). After a final washing step, cells were re-suspended in 100 µl of PBS and 7 µl of each sample were plated on MH plates and incubated overnight at 37°C to confirm inactivation of the bacteria. All data were collected using a BD FACS CANTO II (BD Bioscience) by acquiring 10,000 events, and data were analyzed using the Flow-Jo software (v.8.6, TreeStar Inc.).

7.6. Serum Bactericidal Assay (SBA)

Serum bactericidal activity against *N. meningitidis* strains was evaluated as previously described (Giuliani, M.M. et al. 2005) with pooled baby rabbit serum (Cederlane) used as the complement source (rSBA). Bacteria were grown in Mueller Hinton broth (MH), plus 0.25% (w/v) glucose and the indicated concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma) at 37°C (or 30°C as specified for respective experiments) with shaking until early exponential 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. The incubation with 25% of baby rabbit complement with or without polyclonal mouse sera at different dilutions was performed at 37°C (or 30°C as specified for respective experiments) with shaking, for 60 min. Serum bactericidal titers were defined as the serum dilution resulting in a at least 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.

7.7. RNA stability assay

The *N. meningitidis* NGH38 wild type strain was grown at 30°C or 37°C until mid-exponential phase and active transcription was stopped by adding rifampicin (200 ug ml^{-1}). Each culture was kept at 30°C or 37°C for 15 min by using a pre-warmed water bath. Three ml of culture were collected at different timepoints (1,2 and 5 minutes) and processed for total RNA isolation. Whole cell protein extracts were taken at different time points and analyzed by Western blotting.

7.8. Protein stability assay

The *N. meningitidis* MC58 wild type strain was grown at 30°C or 37°C until mid-exponential phase and active translation was stopped by adding spectinomycin (150 ug ml^{-1}). Each culture was split in two equal parts and incubated at either 30°C or 37°C for 2 h. Whole cell protein extracts were taken at different time points and analyzed by western blotting.

7.9. nOMV vaccine preparation

The nOMV vaccines were prepared growing 5/99 derivative strains in MCDMI - Meningitidis Chemical Define Medium I- medium. The *N. meningitidis* strains were first pre-inoculated into 7 mL of MCDMI at an OD_{600} ranging from 0.15-0.2 and incubated at 37°C at 180 rpm until mid-exponential phase (=0.8-0.9 OD). Due to the stable nature of the chromosomal integration, no antibiotics were added to the growth medium. The mid-exponential pre- cultures were inoculated into 50 mL medium in 250 mL baffled flasks and grown over night (16-18 h) until late stationary phase at 37°C and 180 rpm. BL21(DE3) ΔTolR strains were pre-cultured overday in 7 ml of LB medium supplemented with 100 µg/ml of ampicillin. The cultures were diluted 1:100 in 50 ml of HTMC liquid medium and incubated overnight at 30°C, 5% CO_2, 160 rpm. Bacteria were centrifuged at 3400 × *g* for 15 min, resuspended into 60 ml of fresh HTMC broth supplemented with 1mM IPTG and incubated for 3 h at 37°C, 5% CO_2, 160 rpm. The bacterial cells were pelleted by centrifugation for 30 min at 3400 × *g* and 4°C and the culture supernatants filtered (pore size 0.22 µm). nOMV were collected from the filtered supernatants by ultracentrifugation for 2 h at 96000 × *g*, 4°C and an additional washing step with 1X PBS was performed. The nOMV pellets were re-suspended depending on their size in 50-200 µl of 1X PBS. Protein concentrations were determined by a microtitter plate-based Lowry protein assay (DC protein assay, BioRad).

Table 7.1 Growth medium MCDMI (Meningitidis Chemical Define Medium I) recipe

Component	g l^{-1}	mM
Soy peptone (Bacto Soytone)*	15	NA
Sodium chloride	5.8	99.2
Potassium sulfate	1	5.7
Potassium phosphate dibasic	4	23.0
L-glutamic acid	5	34.0
L-arginine	0.3	1.7
L-serine	0.5	4.8
L-cysteine	0.23	1.9
Magnesium chloride hexahydrate	0.41	2.0
Calcium chloride**	0.021	0.189
Ferric citrate***	0.002	0.008
Glycerol****	5	NA

* *Neisseria meningitidis* will not grow in all Soypeptone extracts! Use Bacto Soytone, Catalog number 243620, BD Biosciences.

** $CaCl_2$ stock solution, 10 g l^{-1} (0. M), 2.1 ml

*** Ferric citrate stock solution, 0.5 g l^{-1}, 4.0 ml

**** Glycerol serves to stabilize OMVs

Table 7.2 Growth medium HTMC recipe

Component	g l^{-1}
Glycerol	15 g/l
Yeast Extract	30 g/l
$MgSO_4$ $7H_2O$	0.5 g/l
K_2HPO_4	20 g/l
KH_2PO_4	5 g/l

*pH 7.35 with KOH 1M

7.10. Transmission Electron Microscopy

Negative Stain Electron microscopy

Purified OMVs were diluted to 0.03 mg/ml in PBS, pH 8 buffer and 5 µl were loaded for 3 minutes onto a 300 mesh carbon/ formvar coated copper grid (Agar Scientific). Blotted the excess, the grids were negatively stained with 1% aqueous Uranyl Acetate for 45 seconds and anlyzed using a Tecnai G2 Spirit transmission electron microscope operating at 100 kV equipped with a Morada CCD camera. The micrographs were acquired at a magnification of 87000 X.

Immunogold electron microscopy

Purified OMVs were diluted to 0.03 mg/ml in PBS pH 8 buffer and 5 µl were loaded for 3 minutes onto a 300 mesh carbon/ formvar coated nickel grids (Agar Scientific). Blotted the excess, the grids were firstly blocked with 0.5%PBS-BSA and then incubated with primary anti-NadA mouse polyclonal serum (diluted 1:4000 in PBS

with 0.5% bovine serum albumin) for 1 h. Grids were washed several times and incubated with 5- or 10-nm gold-labeled anti-mouse secondary antibody (diluted 1:2000 in in PBS with 0.5% bovine serum albumin) for 1 h. After several washes with distilled water the grids were negatively stained in 1% uranyl acetate and observed using a TEM FEI Tecnai G2 spirit microscope operating at 100kV and equipped with an 2K × 4K CCD Olympus SIS Morada camera (Olympus, Shinjuku, Tokyo, Japan). Images were acquired and processed using iTem (OSIS, Olympus, Shinjuku, Tokyo, Japan) software.

nOMV were fixed overnight in 2.5% glutaraldehyde in PBS and then washed and re-suspended in the same buffer. A drop of suspension was placed on formvar/carbon-coated grids, and nOMV were adsorbed for 5 min. Grids were then washed with distilled water and blotted with a filter paper. For negative staining, grids were treated with 2% uranyl acetate for 1 min, air-dried and viewed with a Joel JEM 1200EXII electron microscope operating at 80 kV.

7.11. Mice immunizations

Groups of five between 6- to 8-week old female CD-1 mice (Charles River) were immunized intra-peritoneally (IP). For each injection, the mice received a total dose of 5 µg of nOMV vaccines or 20 µg of NadA recombinant proteins. The nOMV or the recombinant protein vaccines were absorbed with aluminum hydroxide (Alum, 3 mg ml^{-1}) and administered in three doses at day 1, 21 and 35. Blood was taken at day 0, 20, 34 for further analysis and bleed out was performed on day 49. The experiment complied with the relevant guidelines of Italy and the institutional policies of GSK Vaccines.

7.12. IgG antibody titers elicited against NadA

Serum antibody titers against NadA were measured by ELISA, which was performed as described elsewhere (Welsch, J.A. et al. 2003). Microtitter plates were coated overnight at 4°C with 0.015 µM of purified NadA recombinant protein variant 3. Plates

were incubated with single mice sera followed by alkaline phosphatase-conjugated anti-mouse antibodies. After addition of p-nitrophenyl phosphate, optical density was analyzed using a plate reader at a dual wavelength of 405/620-650 nm. Antibody titers were quantified via interpolation against a reference standard curve.
To quantify the IgG subclasses, the microtitter plates were coated overnight at 4°C with 1.0 µg ml^{-1} of purified NadA recombinant protein variant 3, following the protocol described above. Finally, the antibody titers were quantified as the dilution of serum that gave an absorbance of 0.4 OD.

7.13. Inhibition of the binding assay

Escherichia coli BL21(DE3)$T1^R$ (New England Biolabs) was used to express full-length NadA variant 3 following transformation with pET21-*nadA*, as previously described (Capecchi, B. et al. 2005), or the empty vector as control. *E. coli* was cultured at 37°C in Luria–Bertani (LB) broth supplemented with 100 µg/mL of ampicillin. Surface protein expression for full-length NadA was achieved without addition of Isopropil-β-D-1-tiogalattopiranoside (IPTG) exploiting the expression due to the leakage in the induction system. Chang epithelial cells (Wong-Kilbourne derivative, clone 1-5c-4, human conjunctiva, ATCC CCL-20.2) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 10% heat-inactivated fetal bovine serum (iFBS). Cells were grown at 37°C in 5% CO_2.

Chang cells were seeded in a 24 well plate (1.5 x 10^5 cells/well) in antibiotics-free medium with 1% iFBS (Infection Medium) and were allowed to grow for 24 h. For adhesion analysis, *E.coli* wild type or expressing NadA were added at a multiplicity of infection (MOI) of 100 and allowed to adhere for 3 h at 37°C and 5% CO_2. Unattached *E. coli* were removed by extensive washing, and bacteria were counted using serial dilution plating. For inhibition of binding assay, recombinant *E. coli* wild type or expressing NadA were pre-incubated in rotation with diluited sera (1:20; 1:200; 1:2000) or infection medium alone for 1 hour at 4°C before addition to the cells.

8. Appendix

Table 8.1 Plasmids and strains used in this study

Plasmid or strain	Description	Antibiotic resistance[a]	Reference or source
Plasmids			
pBluescript (pBS)	Cloning vector	Amp	Stratagene
pBS-UD*gna2132*	Plasmid for deletion of *N.meningitidis nhba* gene by homologous recombination	Amp, Ery	(Serruto, D. et al. 2010)
pBS_*nhba*	pBS derivative for in locus complementation of *nhba* gene (MC58 sequence)	Amp, Cml	(Vacca, I. et al. 2016)
pComCmr-P_{ind}	Plasmid for allelic replacement at chromosomal location between ORFs NMB1428 and NMB1429 and inducible expression under control of P_{tac} promoter and *lacI* repressor	Amp, Cml	(Ieva, R. et al. 2005)
pCOM-C*nhba*	Plasmid for complementation of *nhba* null mutant. Derivative of pComCmr-P_{ind} containing a copy of *nhba* (MC58 sequence)	Amp, Kan	(Serruto, D. et al. 2010)
pCOM-Ptac_*nhba*	Plasmid for complementation of *nhba* null mutant. Derivative of pComCmr-P_{ind} containing a copy of *nhba* (MC58 sequence)	Amp, Cml	(Serruto, D. et al. 2010)
pBS_14aa_*mCherry*	Derivative of pBS_*nhba*. Construct for complementation of the *mCherry* reporter gene under the control of *nhba* intergenic region	Amp, Clm	This study
pCOM_14aa_*mCherry*	Construct for complementation of the *mCherry* reporter gene under the control of *nhba* intergenic region. Derivative of pComCmr-P_{ind}	Amp, Clm	This study
pCOM_Ptac_*mCherry*	Construct for complementation of the *mCherry* reporter gene under the control of an IPTG-inducible Ptac promoter. Derivative of pComCmr-P_{ind}	Amp, Clm	This study
pUClpxL1kanR	Construct for generating knockout of *lpxL1* gene	Kan	(Koeberling, O. et al. 2008)
pET21b	*E.coli* expression vector	Amp	Novagen
pET21b_NadAv3	pET21b derivative. Construct for NadAv3 expression	Amp	(Capecchi, B. et al. 2005)
pD1843ko::AmpR,	pGEM-T (Invitrogen) derivative.	Amp, Clm	(Fagnocchi,

CmR	Construct for generating knockout of the NadR gene		L. et al. 2012)
pDNadA::EryR	pBS derivative. Construct for generating knockout of the *NadA* gene	Ery	(Fagnocchi, L. et al. 2012)
N.meningitidis strains			
M11205	Clinical isolate		
M11205 Δ*nhba*	*nhba* null mutant of M11205,	Ery	This study
M11822	Clinical isolate		
M11822 Δ*nhba*	*nhba* null mutant of M11822,	Ery	This study
NGH38	Clinical isolate		
NGH38 Δ*nhba*	*nhba* null mutant of NGH38,	Ery	This study
MC58	Laboratory-adapted *N.meningitidis* reference strain		
MC58 Δ*nhba*	*nhba* null mutant of MC58,	Ery	(Serruto, D. et al. 2010)
M10935	Clinical isolate		
M10935 Δ*nhba*	*nhba* null mutant of M10935,	Ery	This study
M14933	Clinical isolate		
M14933 Δ*nhba*	*nhba* null mutant of M14933,	Ery	This study
M10713	Clinical isolate		
M10713 Δ*nhba*	*nhba* null mutant of M10713,	Ery	This study
M03279	Clinical isolate		
M03279 Δ*nhba*	*nhba* null mutant of M03279,	Ery	This study
N16/97	Clinical isolate		
N16/07 Δ*nhba*	*nhba* null mutant of N16/07,	Ery	This study
M11204	Clinical isolate		
M11204 Δ*nhba*	*nhba* null mutant of M11204,	Ery	This study
M10282	Clinical isolate		
M10282 Δ*nhba*	*nhba* null mutant of M10282,	Ery	This study
M07-0240679	Clinical isolate		
M07-0240679 Δ*nhba*	*nhba* null mutant of M07-0240679,	Ery	This study
M11719	Clinical isolate		
M11719 Δ*nhba*	*nhba* null mutant of M11719,	Ery	This study
M16453	Clinical isolate		
M16453 Δ*nhba*	*nhba* null mutant of M16453,	Ery	This study
M18070	Clinical isolate		

M18070 Δ*nhba*	*nhba* null mutant of M18070,	Ery	This study
8047	Clinical isolate		
MC58 Δ*nhba*-C_*nhba*	Complemented *nhba* mutant of MC58	Ery, Kan	(Serruto, D. et al. 2010)
MC58 Δ*nhba*-Ptac_*nhba*	Complemented mutant of MC58 expressing *nhba* (MC58 sequence) under the control of an IPTG-inducible Ptac promoter	Ery, Clm	(Serruto, D. et al. 2010)
MC58 Δ*nhba*-14aa_*mCherry*	Complemented mutant of MC58 expressing *mCherry* reporter gene under the control of *nhba* intergenic region (MC58 sequence)	Ery, Clm	This study
MC58 Δ*nhba*-Ptac_*mCherry*	Complemented mutant of MC58 expressing *mCherry* reporter gene under the control of an IPTG-inducible Ptac promoter	Ery, Clm	This study
5/99	Clinical isolate		
5/99 ΔlpxL1	*lpxL1* null mutant of 5/99	Kan	This study
5/99 ΔlpxL1 Δ*NadA*	Derivative of 5/99 ΔlpxL1; NadA null mutant	Kan, Ery	This study
5/99 ΔlpxL1	Derivative of 5/99 ΔlpxL1; NadR null mutant	Kan, Cml	This study
E.coli strains			
DH5-α	supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1		(Hanahan, D. 1983)
BL21(DE3) ΔTolR	hsdS gal (λcIts857 ind1 Sam7 nin-5 lacUV5-T7 gene 1)		(Berlanda Scorza, F. et al. 2008)
BL21_NadAv3	BL21(DE3) derivative strain carrying the pET21b_NadAv3 plasmid for expression of NadA	Amp	This study

[a]Amp, ampicillin; Kan, kanamycin, Ery, erythromycin; Clm, chloramphenicol

Table 8.2 Oligonucleotides used in this study

Oligonucleotide	Sequence[a]	Restriction site	Application	Reference or source
NMB2132RT-F	GGCTTGTATTTTTGCCCTTTC		qRT-PCR *nhba* gene	This study
NMB2132RT-R	GCATCTTCCTTTGCCTCTGT			
mCherry_RT1 fw	GCTCAGTTTCAGGTAGTCCG		qRT-PCR *mCherry* gene	This study
mCherry_RT1 REV	AGCCCTCAATTCATGTACGG			
pRTNM16sII.F1	GTGGGGAATTTTGGACAATG		qRT-PCR 16s gene	This study
pRTNM16sII.R1	CAACAGCCTTTTCTTCCCTG			
mCherry Fw	TCCAAAGGCGAGGAAGATAAC		Generation of Pwt_mCherry fusion: insert PCR to amplify the *mCherry* gene optimized for *N. meningitidis*	This study
mCherry Rev	CACGGATCCCTGCAGTTATTTGTACAGTTCGTCCATG			
CmR Fw	CTGCAGGGATCCGTGATATAG		Generation of Pwt_mCherry fusion: vector PCR on pBS_*nhba* background	This study
Pwt_14aa Rev	CTTCCTCGCCTTTGGAGGCAAAAATACAAGCCATTGC			
Insert Fw	CGAAAGTGGGAATCTAGACATTATCGGCGTGATTCAG		Subcloning of Pwt_mCherry fusion into pCOM plasmid	This study
Insert Rev	GAGCATAAAATTTTAGTAACCTATGTTTTTATTCAGCAAGTCTTGTAATTC			
Vector Fw	GAATTACAAGACTTGCTGAATAAAAACATAGGTTACTAAAATTTTATGCTC		Subcloning of Pwt_mCherry	This study

Vector Rev	CTGAATCACGCCGATAATGTCTAGATTCCCACTTTCG		fusion into pCOM plasmid	
NmmCh_NdeI Fw	GAAACACATATGATGGTGTCCAAAGGCGAG	NdeI	Cloning mCherry gene into pCOM_Ptac plasmid	This study
NmmCh_NsiI Rev	GTGTCAATGCATTTATTTGTACAGTTCGTCCATGCC	NsiI		
Mut_1_Fw	GAAATACGATGAAGGAGATGATGTTTAAACGCAGCG		Deletion of putative TSS	This study
Mut_1_Rev	CGCTGCGTTTAAACATCATCTCCTTCATCGTATTTC			
Mut_2_Fw	GAAATACGATGAAGGAGATGTTTAAACGCAGCG		Deletion of putative TSSs	This study
Mut_2_Rev	CGCTGCGTTTAAACATCTCCTTCATCGTATTTC			
Mut_3_Fw	ACCAAAAAGGAAATACG**CTG**AAGGAGATGATGATGTT		Mutation of putative TSS	This study
Mut_3_Rev	AACATCATCATCTCCTT**CAG**CGTATTTCCTTTTTGGT			
Mut_4_Fw	AAAGGAAATACGATGAAGGAG**CTGCTGCTG**TTTAAACGCAGCGTAATCGCA		Mutation of putative TSSs	This study
Mut_4_Rev	TGCGATTACGCTGCGTTTAAA**CAGCAGCAG**CTCCTTCATCGTATTTCCTTT			
Mut_5_Fw	GCAATGGCTTGTAT**C**TTTGCCCTTTCAGCCTGC		Site-directed mutagenesis of T-rich region	This study
Mut_5_Rev	GCAGGCTGAAAGGGCAAA**G**ATACAAGCCATTGC			
Mut_6_Fw	CAATGGCTTGTATTTT**C**GCCCTTTCAGCCTGCG		Site-directed mutagenesis of T-rich region	This study
Mut_6_Rev	CGCAGGCTGAAAGGGC**G**AAAATACAAGCCATTG			
Mut_7_Fw	CGCAATGGCTTGTAT**C**TT**C**GCCCTTTCAGCCTGCG		Site-directed mutagenesis of T-rich region	This study
Mut_7_Rev	CGCAGGCTGAAAGGGC**G**AA**G**ATACAAGCCATTGCG			
NMB1368_FwEst	TCGTATTCCTGAAGGTGGATTCGATCGCCGTAAACATCG		Deletion of NMB1368	This study
NMB1368_RevInt_BamHI	GTGCTGAAACATTGCAAAAAGGATCCCTATCCTTGGAAGACAACAA	BamHI		

NMB1368_FwInt_BamHI	TTGTTGTCTTCCAAGGATAGGGATCCTTTTTGCAATGTTTCAGCAC	BamHi		
NMB1368_RevEst	ACATTGTTATGTTGCCGTTTGATTTTCAGACGGCATTTTGTTT			
NMB0838_FwEst	TGCATGACTTGTTGTTGTTTGGTTTGGGCAATATCGCGCGTGT			
NMB0838_RevInt_BamHI	AAATATACATTTGACCGTAGGATCCATCCTGCTTAACCGTCTTTTTA	BamHI	Deletion of NMB0838	This study
NMB0838_FwInt_BamHI	TAAAAAGACGGTTAAGCAGGATGGATCCTACGGTCAAATGTATATTT	BamHI		
NMB0838_RevEst	GTGGAAAACGGCGTGATTGCCTACCCCGTCCACGAGATTA			
2132 mRR1	GCGCGATTT**G**GG**G**GTTCTGCA**G**GGTCG**G**GG**G**GGTCGCTTCC		Mutation of Arg-rich motif in Gly stretch	(Serruto, D. et al. 2010)
2133 mRR2	GGAAGCGACC**C**CC**C**CGACC**C**TGCAGAAC**C**CC**C**AAATCGCGC			
ΔTFSAmRR Fw	TCTTTTATAAACCTAAACCCCGATTTAGGCGTTCTGCACGGT		Deletion of NalP target domain sequence	This study
ΔTFSAmRR Rev	ACCGTGCAGAACGCCTAAATCGGGGTTTAGGTTTATAAAAGA			
LpxL1_ext fw	GGCGGTTTGAGTTAGGAAGC		Check integration in *LpxL1* locus	This study
LpxL1_ext rev	CGTTTGAAAACAAAGTGGCAAAGGC			
Kan Int_fw	ATTATCGAGCTGTATGCGGAGTG		Check integration in *LpxL1* locus	This study
Kan Int_rev	GCAATCCACATCGGCCAGAT			
ΔNadRExt_Fw	CAATATGCCCGTGCAGGT		check ΔNadR deletion	This study
ΔNadRInt_Rev	ATCGAGGATGCGTTGAACTC			
ΔNadRInt_Fw	AAG GGA TTA CAT CGG CAG			
ΔNadRExt_Rev	GGG ACA GTT TAG CCG AGA			
ΔNadAExt_Fw	CGATATGGACGTCGACGTCCTCGAT		check ΔNadA deletion	This study
ΔNadAInt_Rev	GTGGTTTCATCCAGAGCGGCATCAG			
ΔNadAInt_Fw	TCGGTTCAATGTAACGGCTGCAGT			
ΔNadAExt_Rev	TTGACGGAAATCGGCATTACGGGC			
ΔNMB0838_Fwext	TATGCACCATCGCCCGTTGCAT		check ΔNMB0838 deletion	This study
ΔNMB0838_RevExt	ACAACCTGTATTTGAACCATACGCACGA			

ΔNMB0838_RevInt_CmrR	TTACGTTTGGGAAGTATTATGAGGA	check ΔNMB0838 and ΔNMB1368 deletion	This study
ΔNMB0838_FwInt_CmrR	CTATCCACTATATCATAAATCTATCCAC	check ΔNMB0838 and ΔNMB1368 deletion	This study
ΔNMB1368_FwExt	TTCTCGTACTGCTGCAAACCTT	check ΔNMB1368 deletion	This study
ΔNMB1368_RevExt	AAATTCACACGGGCAGGCAACCAA		
pRTNM4	GTTTTGACAGCGTGTCCG		This study
pRTNM5	CAGGTTCTCAAGGACAGGGC		This study
pRTNM6	CGCCTTTACAGTGGGTCAAC		This study
pRTNM7	GTTTACCTGCCTGCGTGA		This study
pRTNM8	GAAGAGTTCAGCGCAGTTTC		This study
pRTNM9	CTGCTGATTCCTTTGGC		This study
pRTNM10	CCTGTCCTTGAGAACCTG		This study
pRTNM11	GTTGACCCACTGTAAAGGC	Sequencing of NHBA mutants	This study
pRTNM12	CGAAGGGCATACGATCC		This study
pRTNM13	ATTACGGTTGACTTCGCCAC		This study
pRTNM14	AATCACGCCGATAATGAAGC		This study
pRTNM15	AAATCGCTGCTGATGAACCT		This study
pRTNM16	GTAACGTCCGAACCGTGATT		This study
pRTNM17	CTTCGACCGACGTTTTGTTT	Sequencing of NHBA mutants (intergenic region)	This study
pRTNM18	AAAATATGCCGTCTGAACGC		This study
pRTNM19	GCGTTCAGACGGCATATTTT		This study
pRTNM20	CAGGCTGAAAGGGCAAAA		This study
pRTNM21	TATTTTTGCCCTTTCAGCCT	Sequencing of NHBA mutants	This study

pRTNM22	CGCCATTGCCTGTATTTTCT	Sequencing of NHBA mutants (intergenic region)	This study
pRTNM23	ATACGCTGATTGTCGATGGG		This study
pRTNM24	ATTTTTCCGCCCCGTAAGT	Sequencing of NHBA mutants	This study
COMseq	TTTGAAAATGAGATTGAGC	Sequencing of NHBA mutants	This study
ComCFw	CCTCGAGCCGCTGACCGAAGG	Complementation check in the COM region (upstream recombination)	This study
ComRev	ACCGGCATCGGCAACTACAC	Complementation check in the COM region (downstream recombination)	This study
CM-UP-C	GGTCGAAATACTCTTTTCGTGTCC	Complementation check in the COM region (upstream recombination)	This study

[a]Underlined letters indicate restriction enzyme sites, bold letters indicate nucleotide mutationa

Trademark statement

Bexsero is a trademark of the GSK group of companies.

Sponsorship, Funding and Conflict of Interest

This work was sponsored by Novartis Vaccines, now acquired by the GSK group of companies. SB has received PhD fellowship from GSK Vaccines srl.

Acknowledgments

I wish to thank Isabel Delany at GSK Vaccines in Siena, for her precious help and support during the PhD program. I would like to thank also Vincenzo Scarlato from University of Bologna for his support and his thoughtful feedbacks and discussion.

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