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The role of the NadR regulator during infection and its implication for the coverage of a new Meningococcus B vaccine

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ATTIVITÀ DI RICERCA

Durante il Dottorato di ricerca mi sono occupato dello studio della regolazione dell´espressione genica in *Neisseria meningitidis.* In particolare ho studiato la regolazione trascrizionale mediata dal repressore NadR, in risposta a segnali presenti nell'ospite e di rilevanza fisiologica. Ho caratterizzato il meccanismo molecolare di repressione mediato da NadR sui suoi geni target e il meccanismo mediante il quale molecole presenti nei siti d'infezione del meningococco alterano l'attività regolatoria di NadR. Infine, ho studiato l'impatto della regolazione mediata da NadR su l'antigene NadA, sulla copertura di un nuovo vaccino meningococcico, chiamato 4CMenB.

In parallelo, ho indagato il ruolo nella regolazione trascrizionale di piccoli RNA non codificanti indotti in diverse condizioni e/o stress incontrati dal meningococco durante la sua patogenesi.

Nel periodo del Dottorato di Ricerca sono stato co-autore dei seguenti lavori scientifici:

Fagnocchi L, Pigozzi E, Scarlato V, Delany I. "In the NadR regulon, adhesins and diverse meningococcal functions are regulated in response to signals in human saliva". J Bacteriol. 2012 Jan;194(2):460-74. doi: 10.1128/JB.06161-11. Epub 2011 Nov 11.

Brier S, **Fagnocchi L**, Donnarumma D, Scarselli M, Rappuoli R, Nissum M, Delany I, Norais N. "Structural Insight into the Mechanism of DNA-Binding Attenuation of the Neisserial Adhesin Repressor NadR by the Small Natural Ligand 4-Hydroxyphenylacetic Acid". Biochemistry. 2012 Aug 28;51(34):6738-52. Epub 2012 Aug 15.

Fagnocchi L, Biolchi A, Ferlicca F, Boccadifuoco G, Brunelli B, Brier S, Norais N, Chiarot E, Bensi G, Kroll JS, Pizza M, Donnelly J, Giuliani MM, Delany I. "Transcriptional Regulation of the *nadA* Gene in *Neisseria meningitidis* Impacts on the Prediction of Coverage of the 4CMenB Vaccine". Infect Immun. 2013 Feb;81(2):560-9. doi: 10.1128/IAI.01085-12. Epub 2012 Dec 10.

Fagnocchi L, Bottini S, Fantappiè L, Golfieri G, del Tordello E, Siena E, Serruto D, Scarlato V, Muzzi A, Delany I. "Global identification and characterization of small non-coding RNAs in *Neisseria meningitidis* in response to multiple stress conditions". Manuscript in preparation.

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1 INTRODUCTION

1.1 Meningococcal disease

Neisseria meningitidis, otherwise known as meningococcus, is an exclusively human pathogen, which represents a major cause of meningitis and sepsis, devastating meningococcal disease, which can kill children and young adults within hours despite the availability of effective antibiotics.

Meningococcal disease was first reported in 1887 by Anton Weichselbaum, who described the meningococcal infection of the cerebrospinal fluid of a patient [1]. Each year there are an estimated 1.2 million cases of invasive meningococcal disease and 135,000 deaths. During epidemics the incidence of meningococcal disease can rise above 1 per 1,000 persons [2, 3]. Despite availability of antibiotic treatment, approximately 10 to 14% of people who contract meningococcal disease die, with a rate between 40-55% among patients with meningococcal sepsis [4, 5]. Approximately 11 to 19% of individuals surviving the disease often suffer from permanent sequelae, including neuro-developmental deficits, hearing loss, seizures, ataxia, hemiplegia as well as amputation of limbs [4, 6-8].

Most cases of meningococcal disease occur in otherwise healthy individuals without identified risk factors and for reasons not fully understood. However, certain biological, environmental and social factors have been associated with an increased risk of disease. Infants under 1 year of age, with a peak between 0 and 7 months, are the population at highest risk of infection due to their immature immune systems (6.33-7.08 cases per 100,000). A second peak in incidence is observed in adolescents and young adults (14-24

years; 0.75 cases per 100,000) largely due to increased carriage in this population [9]. Microbial factors influencing its virulence, environmental condition facilitating exposure and acquisition, impaired immune system, genetic polymorphisms and naso- and oropharyngeal irritation caused by smoking and respiratory tract infection, represent important factors for disease development [4, 10-15].

As the classic signs and symptoms, such as rash, fever, and headache, are unspecific mainly in the early course, diagnosis of meningococcal disease becomes challenging and may be mis-diagnosed. Due to the rapid progression of meningococcal disease, if appropriate treatment is delayed and sometimes despite early antibiotic treatment, it can lead to death within 24 to 48 hours from the first sign of symptoms [6].

Meningococcal disease occurs mainly as sporadic cases in industrialized countries, even if small regions suffer from epidemic outbreaks (e.g. New Zealand). On the contrary, it is largely epidemic in the so-called “meningitidis belt” in the sub-Saharan Africa.

1.2 The pathogen

Neisseria meningitidis is a β-proteobacterium, gram-negative, bean-shaped diplococcus (Figure 1.1) and member of the bacterial family of Neissiriaceae. As a Gram negative, it has an outer membrane composed of lipids, outer membrane proteins (OMPs), and lipooligosaccharide (LOS), the peptidoglycan layer and the inner membrane. A polysaccharide capsule is ususllay attached to the outer membrane of meningococcus. Pathogenic strains are almost always encapsulated, however the invasive potential of non encapsulated disease isolates has recently been reported [16]. It is aerobic and requires

glucose, pyruvate or lactate as carbon sources [17], optimal growth occurs at 35-37 °C with 5-10% (v/v) CO_2. It is non-motile and non-sporulating and generally piliated.

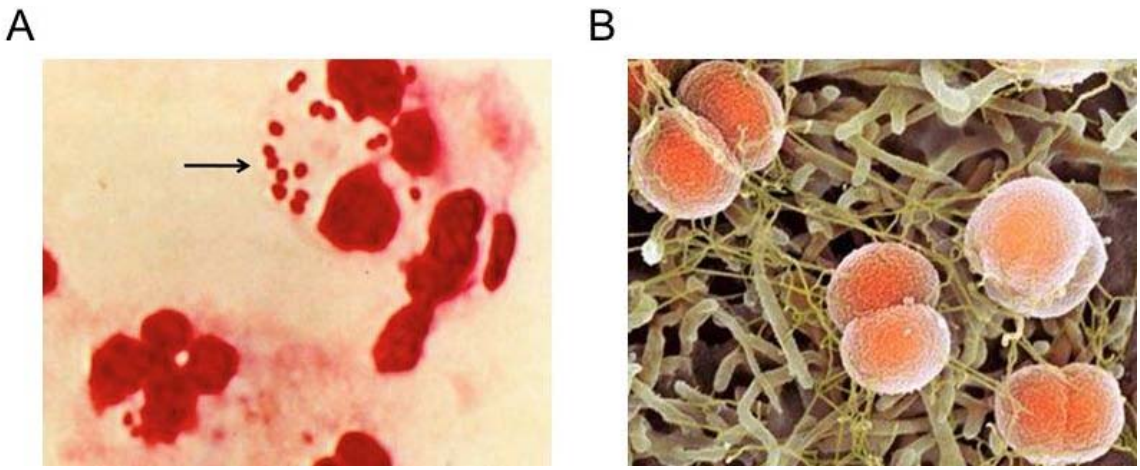


Figure 1.1 - *Neisseria meningitidis* diplococci.

(A) Meningococcal diplococci indicated by the black arrow in proximity and within leukocytes in the cerebrospinal fluid (Gram staining, modified from [18]). (B) Colored scanning electron micrograph (SEM) of *Neisseria meningitidis* bacteria on human epithelium (modified from www.sciencephoto.com).

1.2.1 Classification and epidemiology

Meningococci strains are traditionally classified by serologic typing systems depending on the structural differences and therefore the immunological reactivity of surface exposed epitopes on the outer membrane or the capsule. 10 serosubtypes and 20 serotypes have been defined according to antigenic properties of outer membrane PorA and PorB proteins, respectively [19] as well as 13 immunotypes depending on LOS differences [20, 21]. On the basis of the bacterial polysaccharide capsule, at least 12 different serogroups have been identified (A, B, C, E-29, H, I, K, L, W , X, Y, Z). Out of these, six are responsible for more than 90% of meningococcal disease worldwide: A, B, C, X, Y and W [2, 22-24].

These serogroups are distributed widely and differently from one part of the globe to the other. In Europe, South America and Australia, serogroups B and C predominate, whereas in Asia serogroups A and C are the most common. In North America, most meningococcal disease is caused by serogroups B, C, and Y [25, 26]. In Africa, epidemics occur every 8-10 years and serogroup A, historically the serogroup responsible for the larger epidemics, is responsible for most cases in the “meningitis belt" region, even if serogroup W also causes a substantial proportion of cases [2, 27, 28], (Figure 1.2).

Meningococcal immuno-evasion phenomena such as high frequency phase and antigenic variation of outer-membrane structures as well as horizontal gene transfer enhance the virulence of meningococci by conferring them genotypic and phenotypic diverstiy and there is also evidence of capsular switching [29-31]. For this reason serotyping is not suitable for modern epidemiology. DNA-based approaches have been developed to characterize meningococcal strains including pulsed-field gel electrophoresis (PFGE), multi locus enzyme electrophoresis (MLEE) and PCR [32-35]. A genetic typing system based on polymorphisms in multiple housekeeping genes, called Multi Locus Sequence Typing (MLST), is now the gold standard for molecular typing and epidemiologic studies. MLST characterizes isolates on the basis of the nucleotide sequences of internal fragments of seven housekeeping genes defining their sequence type (ST) [36]. Menigococci can in this way be classified into lineages, termed clonal complexes (CC). A clonal complex is a group of STs that share at least four of the seven loci in common with a central ancestral genotype [37]. Despite huge diversity in meningococcal population, only a minority of these clonal complexes are associated with invasive disease, known as hyper-invasive lineages [38]. ST-1, ST-4 and ST-5 complexes are restricted nearly exclusively to serogroup

A strains, ST-11 is associated both to C and W-135 serogroups, ST8, ST-32 and ST41/44 are associated with serogroup B worldwide together with the more recent ST-269, while ST-23 complex is associated with serogroup Y[39]. Why hyper-invasive meningococcal lineages are more pathogenic than others remains still unknown.

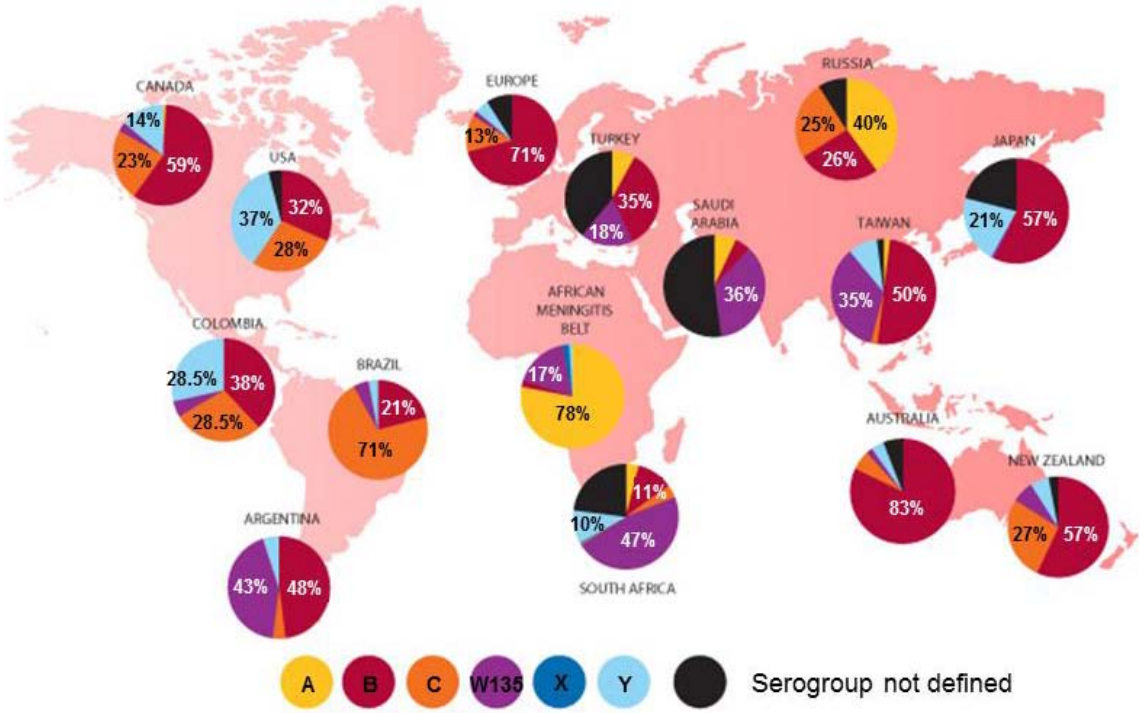


Figure 1.2 - Global distribution of invasive meningococcal serogroups.

The image summarizes the serogroup-specific incidence in different geographical areas of the world (modified from www.meningitisinfo.com).

1.2.2 Pathogenesis

The pathogenesis of *Neisseria meningitidis* is a complex multi-stage process (Figure 1.3). Meningococci may be acquired via respiratory droplets or saliva. The asymptomatic meningococcal colonization of the upper respiratory tract is common and is found in approximately 10-15% of healthy adults [2]. This carrier state represents a successful

commensal relationship between the host and the bacterium: it provides the only known reservoir for the human-adapted meningococcal infection and may also contribute to establishing host immunity [40]. Meningococcal carriage is very low in the first years of life (4.5% in infancy), whereas it is highest in adolescents and young adults, peaking at 10-35% in 20-24-year olds [41, 42] before decreasing to about 8-10% in older than 50 years of age [41, 43]. Compared with the carriage rate, meningococcal disease is rare; however in a small subset of cases the colonization represents the initial step of disease.

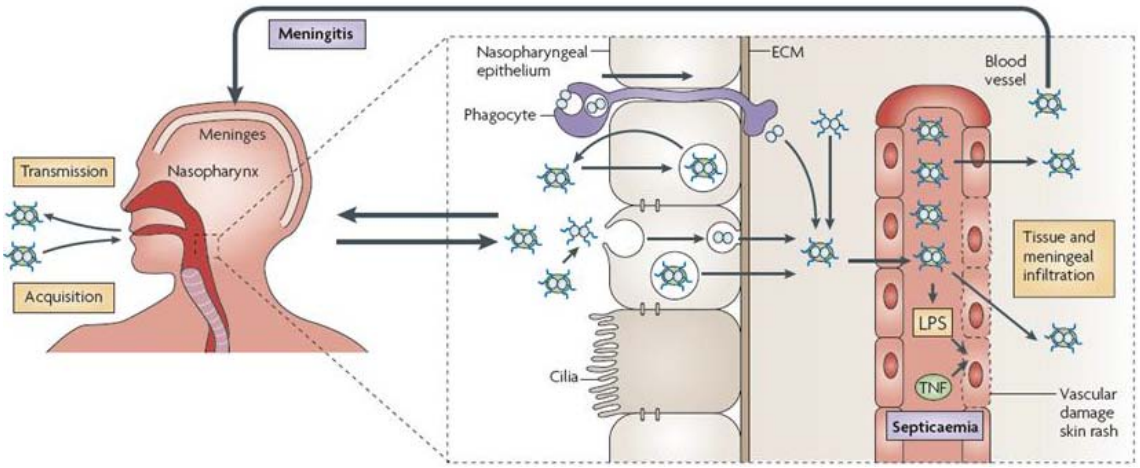


Figure 1.3 - Stages in the pathogenesis of *Neisseria meningitidis*.

The image summarizes the steps during meningococcal colonization and infection. Detailed description is reported in the text. Modified from [44].

The initial contact with nasopharyngeal epithelial cells is mediated by Type IV pili, which may recognize the host receptor CD46 [45], but this remains controversial. Then, meningococci proceed to proliferate on the surface of human non-ciliated epithelial cells, forming small micro-colonies at the site of initial attachment [40]. After the initial colonization, there is a loss or down regulation of the capsule, which sterically masks the outer membrane proteins. This event is thought to occur both via cell contact induced

repression [46], and by selection of low or no-capsule expressing bacteria due to phase variation [47]. Close adherence of meningococci to the host epithelial cells is mediated by a variety of possible redundant adhesins, previously masked by the capsule, resulting in the appearance of cortical plaques [40].

At this stage of colonization meningococci can invade the pharyngeal mucosal epithelium. One trigger of meningococcal internalization is represented by interaction of the bacterial opacity proteins, Opa and Opc, with CD66/CEACAMs and integrins, respectively, on the surface of the epithelial cell [48]. Meningococci can be internalized through the recruitment of factors leading to the formation and extension of epithelial cell pseudopodia that engulf the bacteria within intracellular vacuoles [49, 50]. Once internalized in the epithelial cells meningococcus can evade the host immune response, find more available nutrients and survive thanks to factors including IgA1 protease, which degrades lysosome-associated membrane proteins [51]. Meningococci are capable of intracellular replication partly due to the capacity of the organism to acquire iron through specialized transport systems, such as the hemoglobin binding receptor (HmbR), transferring binding protein (TbpAB) and lactoferrin binding protein (LbpAB) [52].

In healthy individuals, bacteria that cross the mucosal epithelium are eliminated through serum bactericidal activity. However, in susceptible individuals, meningococcus can occasionally cross the mucosal epithelial barrier through either transcytosis or directly following damage to the monolayer integrity or through phagocytes in a ‘Trojan horse’ manner [44] and eventually enter the bloodstream surviving the host defense mechanism. Survival in blood is promoted by mechanisms such as up-regulation of capsule expression, as it provides resistance to antibiotic- and complement- mediated

killing and inhibits phagocytosis [53], and recruitment of both negative regulators of the complement cascade, such us factor H recruited by factor H-binding protein (fHbp) [54] and NspA [55], and complement regulators, such as C4-binding protein (C4bp), bound by PorA porins [56]. Once inside the blood, the meningococci face two fates: either the bacteria multiply slowly in the blood, eventually passing across the brain vascular endothelium (or the epithelium of the choroid plexus) and seeding local sites, resulting in infection of the meninges and the cerebrospinal fluid [57], or they undergo rapid multiplication in the bloodstream, resulting in clinical features of bacterial septicemia or meningococcemia [4, 58].

Meningococcal disease represents a failed or dysfunctional relationship with the host, and the factors that trigger menicococcal entrance in the blood are not yet fully understood but should be dependent on both the host and pathogen and include impairment of the integrity of the human nasopharyngeal mucosa, the lack of a protective immune response and microbial factors influencing virulence [2, 3, 59].

1.3 Genetics

The genome sequences of many disease strains and carriage strains have been reported, showing that the circular meningococcal chromosome is between 2.0 and 2.2 megabases in size and contains about 2000 genes [60-62]. The meningococcus shares about 90% nucleotide homology with either *Neisseria gonorrhoeae* or the commensal *Neisseria lactamica*. 70% of the genome encodes for essential metabolic functions. 10% of the genome is represented by mobile elements, such as IS elements and prophage sequences [60], leading to DNA transfer between meningococci, gonococci, commensal spp. as well

as other bacteria (e.g. *Haemophilus*) [63]. Except for the IHT-A1 capsule locus, no specific core pathogenome has been identified [64], suggesting that virulence may be dependent on multiple redundant genes, and therefore polygenic in nature. The acquisition of the capsule locus by horizontal gene transfer, possibly from *Pasteurella multocida* or *P. hemolytica*, appears to be a major event in the evolution of the pathogenicity of the meningococcus from an un-encapsulated ancestor [40, 61].

The GC percentage is widely variable along the chromosome with an average of 51.63%, with defined regions of low GC content that likely have been acquired by a relatively recent horizontal gene transfer [65]. These events are common in *N. meningitidis* due to its natural transformation competence [66]. Another characteristic of the meningococcal genome is the abundance of repetitive DNA sequences, polymorphic regions and genetic switches (e.g. slipped-strand mispairing), leading to genetic instability, duplication or deletion of regions of the genome, as well as genetic recombination [63, 67]. About 20% of the genome consists of repeated regions of different natures [68]. In summary, a central characteristic of the genome is its plasticity contributing to the non-clonal behaviour of meningococcus and phenotypic diversity, which allow the bacteria to successfully adapt and invade the host.

1.4 Gene regulation and adaptation to the host environment

During infection, *N. meningitdis* can invade diverse sites within the human host, which represent different niches with respect to nutrients, environmental factors and competing microorganisms. Therefore it 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 [69]. Bacteria have two major and complementary mechanisms for adapting to changes in their environment: changing their genotype (genome plasticity) or altering gene expression, both leading to phenotypic variations.

1.4.1 Genome plasticity

The high natural competence of meningococci is a leading cause of horizontal gene transfer and therefore genome variability. In addition, the above mentioned abundance of repetitive DNA sequences contributes to genome plasticity. The most frequent repeat sequence element is the Neisserial DNA uptake sequence (DUS). There are nearly 2000 copies of this 12-bp sequence, involved in recognition and uptake of DNA from environment [70]. The 20 bp long dRS3 elements, comprising 6 bp terminal inverted repeats, promote both permanent genomic changes, such as insertion and chromosomal rearrangement [61], and recombination with exogenous DNA [71]. Correia elements, (CEs), are mobile elements of 100-150 bp, comprising 26 bp inverted repeats, which carry transcription initiation sequences as well as binding sites for DNA bending protein, suggesting a role in modulating the expression of nearby genes [72, 73]. Finally the meningococcal genome is also littered with insertion sequences (IS) [74] and other repeat sequences with incompletely determined functions such as, AT-rich repeats [60] and REP2 repeats [75].

Phase variation (PV). PV is the adaptive process by which bacteria undergo frequent and reversible phenotypic changes resulting from genetic alterations in specific loci of their genomes. Short tandem sequence repeats are the basis for PV, which can occur during replication through slipped-strand mispairing (SSM, Figure 1.4), altering the unit number

of these repeats. The presence of repeat units may cause a slippage during replication of either the synthesis strand, generating addition events, or the template strand, leading to deletions in the new born filament [63]. When repeats occur in the coding sequence, close to or in the promoter region, they can change the transcriptional and translational state of the gene, by introducing frameshift mutations or changing critical promoter spacing. This results in either an on/off switching or an altered level of the gene expression and hence function and antigenicity of the encoded proteins [47, 76-78]. In most loci, known or predicted to be controlled by PV, changes in DNA repeat length produce reversible translational frameshift mutations in the coding sequence of the gene, thereby switching the expression of the encoded protein 'on' or 'off' [79]. On the other hand, the phase variable repeat tracts in the *porA, fetA*, and *opc* genes are located between the -10 and the -35 promoter elements and are thought to result in altered sigma-factor binding [77, 78, 80]. The phase regulation of the Neisserial Adhesin A (NadA), is particular, altering the level of expression of *nadA* and will be discussed below. It has been proposed that in *N. meningitidis* over 100 genes are potentially phase variable, altering mainly virulence-associated, surface-exposed molecules such as outer-membrane proteins PorA, Opc, Opa, pili and specific adhesins, as well as LPS and capsule [63, 81, 82]. It has been demonstrated that meningococcal strains associated with disease have high frequency of phase variation, indicating a substantial benefit in varying surface components during invasion and/or transmission between hosts [83, 84].

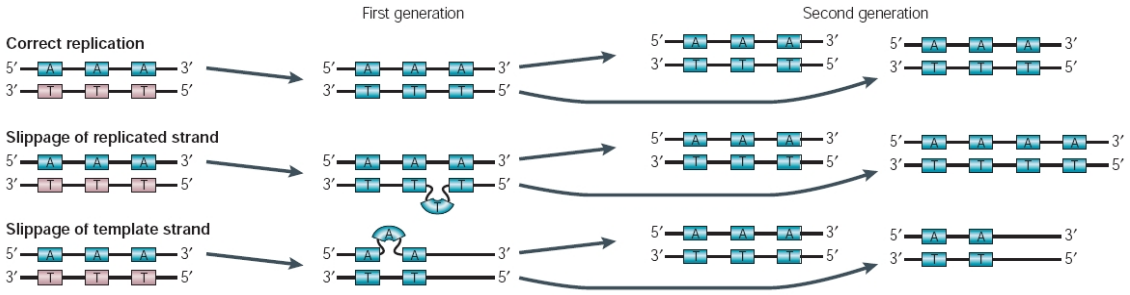


Figure 1.4 - Slipped-strand mispairing.

Slipped-strand nucleotide mispairing can generate variation in gene expression. Illegitimate base pairing in regions of repetitive DNA during replication, coupled with inadequate DNA mismatch repair systems, can produce deletions or insertions of repeat units. Bulging in the replicated and template strands gives rise to larger and smaller numbers of repeat units, respectively. The figure shows a strand of DNA (blue) being carried through two rounds of replication. From [85].

Antigenic variation. Antigenic variation is a mechanism of immune evasion and refers to the expression of functionally conserved moieties that are antigenically distinct within a clonal population [86]. Only one variant is expressed at any given time, although the cell contains the genetic information (or acquires the genetic potential through DNA uptake) to produce a whole range of antigenic variants. This process is distinct from phase variation, as the antigen is consistently produced, but in different forms. In the pathogenic *Neisseria* species, antigenic variation occurs in several surface components, including type IV pili, lipooligosaccharides and opa proteins [63, 87, 88].

1.4.2 Transcriptional regulators

Survival under the extreme and rapidly changing conditions of the host requires timely and appropriate alterations in gene expression and protein activity, which occur in a bacterial cell in response to stimuli signalling these new environmental conditions. At the transcriptional level, these alterations could be controlled by global factors, for example

through changes in associations between different alternative sigma factors and core RNA polymerase, which essentially reprogram promoter recognition specificities of the enzyme to allow expression of new sets of target genes [89]. Although extensive transcriptional regulation is expected to accompany the infection process of *N.meningitidis*, only 36 putative transcriptional regulators (according to the Comprehensive Microbial Resource database, http://cmr.jcvi.org) are encoded by the meningococcal genome, compared to *Escherichia coli*, which harbors more than 200 transcriptional regulators. This reveals a striking limitation for transcriptional regulation, which is possibly related to the restricted ecological niches of the Neisseriacae [90]. Only few of the predicted regulators have been characterized and the regulons of even fewer have been deeply studied, involed in the adaptation of meningococcus to iron and oxygen limitation and response to nitric oxide.

The Ferric Uptake Regulator (Fur). It has been very well established that bacterial pathogenesis and survival are dependent on the ability to acquire iron within the host [91, 92]. Cell growth and multiplication, in fact, require essential nutrients such as iron, which is limiting in the human host being sequestrated by human iron proteins. Although *N. meningitidis* does not produce siderophores for iron acquisition, it possesses outer membrane receptors that have been postulated to scavenge the iron-loaded siderophores secreted by other bacteria colonizing the nasopharyngeal tract [93] such as the hemoglobin binding receptor (HmbR), transferring binding protein (TbpAB) and lactoferrin binding protein (LbpAB) [52]. However, iron overload results in toxicity for the bacterium; therefore, iron uptake is tightly regulated, as in many bacteria, by Fur [94, 95]. Fur senses internal iron concentration and binds to and represses iron uptake genes using

ferrous iron as a co-repressor [96-98]. Fur has been also reported to act positively in the expression of certain genes. The regulon of Fur comprises more than 200 gene [99] regulated either with a direct mechanism (binding upstream promoter sequences [96]) or an indirect mechanism which involves a posttranscriptional regulation mediated by a Fur repressed small regulatory RNA named NrrF [100, 101].

The Fumarate and Nitrate reductase Regulator protein (FNR). During its infection *N. meningitidis* is exposed to highly divergent partial pressures of oxygen (high in the upper respiratory tract and low in the mucus membranes and in the blood [102]. It has been shown that although *N. meningitides* fails to grow under strictly anaerobic conditions, under oxygen limitation the bacterium express a denitrification pathway system that supplements growth [103, 104]. The transcriptional activator FNR enables the meningococci to survive under oxygen limitation by inducing sugar fermentation and denitrification pathways, utilizing nitrite and nitric oxide as electron acceptors [105, 106]. FNR binds to DNA and activates target genes, involved in bacterial anaerobic metabolism as a dimer containing [4Fe-4S] cluster, during oxygen limitation. This cluster dissociates in the presence of oxygen, destabilizing the dimer, with loss of FNR activity [106-108]. Only a total of 9 transcriptional units have been identified as being part of the regulon of FNR [106]. Interestingly factor H binding protein (fHBP) which enables the bacterium to evade complement-mediated killing by binding factor H [54, 109], has been shown to be positively regulated by oxygen limitation through a FNR dedicated promoter [110]. This result, together with the observation that a knock-out of FNR in *N.menigitidis* is attenuated in the mouse and infant rat animal models of infection [106], indicate the

importance of these responses for the pathogenesis and the survival of meningococcus in the human host.

The Nitric oxide (NO)-sensitive Repressor (NsrR). *N. meningitidis* is exposed to the free-radical gas nitric oxide (NO), generated both internally by its own metabolism and externally by the human host tissue in its natural habitat, which is rich in macrophages, a potent source of NO during infection [111, 112]. On one hand, meningococcus synthesizes NO detoxification proteins that protect the organism from being killed by the toxicity of extracellular macrophage-generated NO [113]. On the other hand, under anaerobic growth, it utilizes internally generated NO as part of a bacterial metabolic pathway. NsrR is the major NO-responsive transcriptional regulator, repressing a small regulon of 4 genes. As NO concentration increases, it is specifically inactivated, through the alteration of its iron-sulfur cluster, thus leading to up-regulation of denitrifying genes [114, 115].

Other transcriptional regulators. The LysR-type regulator CrgA is upregulated upon contact with human epithelial cells; it acts as a repressor of its own transcription and reportedly type IV pili subunits [46, 116, 117] and as an activator of the *mdaB* gene, coding for a hypothetical NADPH-quinone oxidoreductase [118].

NMB0573 (annotated as AsnC) is a global regulator controlling the response to poor nutrient conditions, which are perceived by binding of this regulator to leucine and methionine, two amino acids representing general nutrient abundance [119].

The Zinc uptake regulator (Zur) is a Fur-like regulator that responds specifically to Zn and controls Zn-uptake by regulating a TonB receptor that functions in high affinity Zn acquisition [120].

1.4.3 The MarR family of transcriptional regulators

The Multiple antibiotic resistance Regulator (MarR) family of transcriptional regulators is widely distributed throughout the bacterial and archaeal domains and it has been suggested that the MarR family is one of nine families of transcription factors to have evolved before the divergence of these domains over 3 billion years ago [121, 122].

MarR regulators include proteins critical for control of virulence factor production, bacterial response to antibiotics and oxidative stresses and catabolism of environmental aromatic compounds [122-125]. The eponymous protein MarR was originally characterized as the repressor of the multiple antibiotic resistance operon *marAB* in *E. coli* [126]. Typically, MarR-like regulators appear as homodimers and bind to relatively short palindromic or pseudopalindromic binding sites on DNA, consistent with their dimeric structure, although the lengths of the inverted repeats and the spacing between half-sites are variable. Members of the MarR family share the same core fold, in which each monomer consists of 6 α-elices and 2 β-strands (Figure 1.5).

The N-terminal a-helix 1 and the C-terminal helices 5 and 6 interdigitate with the corresponding regions of the other subunit to form a dimerization domain, while the DNA binding domain is represented by wing helix motifs, spanning a-helix 3 and 4 and comprising b-strands 2 and 3. The two DNA binding lobes in the dimer contact the DNA in the major groove [125]. MarR regulators usually act as repressors, even if exceptions are reported, such as BadR from *Rhodopseudomonas palustris* which induces transcription of the *badDEFG* operon [127]. The majority of the MarR family members are regulated by the non-covalent binding of low-molecular-weight specific anionic lipophilic (usually phenolic) compounds.

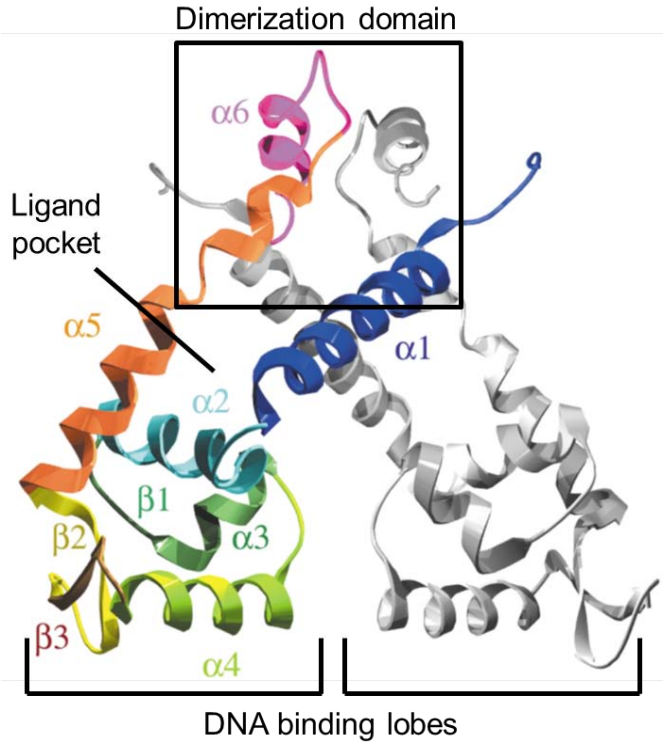


Figure 1.5 - Typical structure of MarR transcriptional regulators.

Structure of *E.coli* MarR dimer, showing the conserved domains of the family of transcriptional regulators. Modified from [128].

The interaction of ligands typically attenuates the ability of MarR dimers to bind their cognate DNA sequence, resulting in de-repression of transcription, but the opposite effect has also been reported [129]. Co-crystal structures of MarR homologs have been obtained with the molecule salicylate which, although a direct biological relevance may not be apparent, binds to numerous MarR homologs albeit at very high concentrations [128, 130-133]. The natural ligands for MarR proteins are often not known. The mechanism of ligand-mediated allosteric control of DNA binding is unique amongst prokaryotic transcriptional regulators in that the DNA and ligand binding domains almost completely overlap in the residues involved [125, 128].

Multiple mechanisms of repression have been described for MarR. The locations of the MarR binding sites often overlap the –35 and/or –10 promoter elements of their target genes, suggesting that repression is achieved by steric inhibition of RNA polymerase binding to the promoter. However, the homolog HpaR from *E. coli* likely represses transcription by blocking promoter escape by RNA polymerase, while SlyA from *Salmonella typhimurium*has been suggested to prevent open complex formation and the binding sites of other homologs suggest that they impede transcriptional elongation [134, 135]. Cooperative binding to closely spaced recognition sequences has been demonstrated for OhrR from *Bacillus subtilis* and may occur for other MarR homologs, as well [136, 137].

A MarR homolog has been characterized from *N. gonorrhoeae* that mediates the resistance of this organism to antimicrobial hydrophobic agents. The Fatty acids resistance Repressor (FarR) represses its own transcription and that of the distally located *farAB* operon, which encodes an efflux pump that exports host-derived antimicrobial agents such as long-chain fatty acids, by binding to three binding sites overlapping and upstream of the *farAB* promoter [138, 139].

Two of the 36 putative transcriptional regulators in *N. meningitidis* are members of the MarR family and are encoded by the genes NMB1585 and NMB1843. The structure of the NMB1585-encoded protein has been resolved, and it was shown to bind to its own promoter DNA, but neither its target genes nor the signal to which it responds is known [140].

1.4.4 The Neisserial adhesin Regulator (NadR)

The product of the NMB1843 gene is a MarR transcriptional regulator of 16.6 kDa per monomer and a homologue (with a nucleotide and amino acids sequences identity of >97% and >99%, respectively) of the gonococcal FarR, which was first described as a regulator of the *farAB* efflux pump that mediates gonococcal fatty acid resistance [138, 139]. In contrast, fatty acid resistance is a high intrinsic feature of *N. meningitidis*, and a knockout of the NMB1843 gene was unchanged in its sensitivity to fatty acids, but adhered considerably more to epithelial cells than the wild type. Therefore, the NMB1843 protein has been reported to play no role in regulating fatty acid and was, instead, demonstrated to repress expression of the meningococcal adhesin NadA [141, 142]. For this reason it was renamed Neisserial adhesin Regulator (NadR).

The urogenital tracts colonized by gonococci are rich in hydrophobic compounds and these bacteria need to rely on active efflux systems for structurally diverse hydrophobic agents [143, 144]. In meningococci, NadR has evolved a different regulatory circuit, since efflux pumps became constitutively expressed. The *nadA* gene was acquired by horizontal gene transfer only by meningococci and, after this event, NadR has gained control over this meningococcus-specific surface protein involved in host colonization, thus contributing to divergent niche adaptation in pathogenic Neisseriae.

As introduced above, *nadA* is phase variable due to a repeat sequence upstream of the *nadA* promoter region, which alters its expression by controlling the transcriptional activity of the promoter [145, 146]. NadR was demonstrated to be the major mediator of this control [141]. It binds with high affinity to two sequences flanking the variable repeat region and changes in the number of repeats affect the ability of NadR to repress the

nadA promoter [141]. 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 [141]. 4HPA is a catabolite of aromatic amino acids and is secreted in human saliva [147]. 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 structural bases of the 4HPA regulation of the NadR DNA binding activity are not clear and represent a topic of this thesis.

NadR is highly conserved throughout all the Neisseria spp., while *nadA* is present only in few strains (see below). For this reason it is logical that NadR should regulate other target genes in addition to *nadA*. It has been described that, even if being a highly specialized repressor of *nadA*, NadR can regulate at lower extent other four genes during exponential growth phase [90]. The identification of genes that may be co-regulated with *nadA* by NadR, in response to signals that could have a biological relevance during pathogenesis of meningococcus, is part of the work of this thesis.

1.5 Virulence factors and adhesins

The virulence of *N.meningitidis* is influenced by multiple factors, comprising both the above mentioned genetic mechanisms, allowing the bacteria to vary its phenotype and adapt to the host, and iron sequestration mechanisms. Additionally, meningococci express multiple molecules acting as endotoxin, secreted factors or surface proteins, located in different compartment of the menincococcal cell membrane (Figure 1.6), which interact with host cellular molecule.

Capsule. The major virulence factor of *N.meningitidis* is the polysaccharide capsule, which plays a crucial role in meningococcal fitness, protecting the bacterium during airborne transmission between hosts [44, 148] and facilitating colonization and virulence by protecting the meningococcus from desiccation and the host innate and adaptive immune effector mechanisms such as phagocytic killing, opsonisation, antimicrobial peptides and complement-mediated bactericidal killing [149, 150]. Like many other virulence factors its expression is phase variable [47] and capsule switching between one serogroup to another provides a selective advantage that allows the bacterium to evade opsonisation or neutralization by natural or vaccine-induced protective anti-capsular antibodies [31].

Lipooligosaccharides (LOS). LOS are endotoxin and 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 [151]. LOS comprises an inner and outer oligosaccharide core attached to the lipid A portion, that anchors the LOS into the outer leaflet of OM. Lipid A is responsible for the toxicity of LOS due to its ability to binds to different receptors on monocytic and dendritic cells triggering the secretion of various inflammation mediators and leading to endothelial damage, capillary leakage and septic shock [152, 153]. Phase and antigenic variations, leading to different saccharide chains, dramatically alter antigenic properties of LOS, enabling individual meningococci to display a repertoire of multiple LOS structures simultaneously [154].

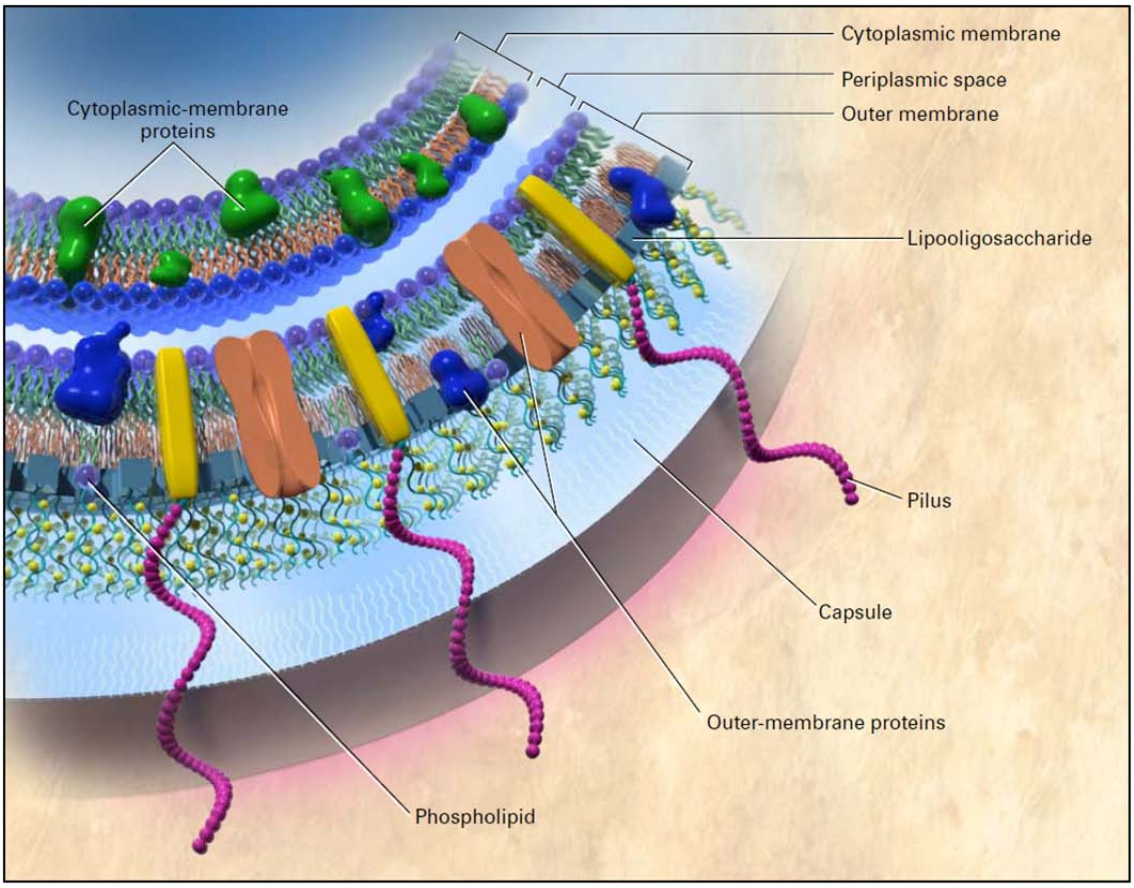


Figure 1.6 - The meningococcal cell membrane.

Cross sectional representation of the different compartments, composing the meningococcal membrane. Major virulence factors are shown and described in the text. From [4].

Pili. Pili are long filamentous structures consisting of protein subunits that extend from the bacterial surface beyond the capsule [155, 156]. Meningococcal pili belong to type IV pilus family, members of which undergo rapid extension and retraction. They represent the major contributor to adhesive property of the capsule and are involved in the initiation of the meningococcus-host cell interaction [157, 158]. In addition to adhesion, pili, are involved in several other functions, such as facilitating the uptake of foreign DNA from the extracellular environment, therefore increasing transformation frequency, a property that contributes to the virulence by promoting genetic adaptability [159].

Twitching motility generated by pilus retraction is important for passage through the mucosal layer, movement over epithelial surface and micro-colonies formation [160]. The pilus is composed of identical subunits of pilin, expressed from the *pilE* locus. The *pilE* gene undergoes sequence variation due to homologous recombination with multiple non-expressed truncated *pilS* genes, resulting in different adhesive and immunogenic pili variants [161].

Opacity proteins (Opa and Opc). 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 [156]. They are beta-barrel proteins, which vary in three of the four surface loops they possess [87]. Both bind the heparansulphate proteoglycans and sialic acids [162, 163] but they also display a degree of receptor specificity for CEACAM (carcino-embryonic antigen-related cell-adhesion molecule), which are highly expressed during inflammation [164, 165].

Minor Adhesins. *N. meningitidis* has evolved a number of other surface structures that mediate interaction with host cells. Numerous apparently minor adhesins are generally expressed at low levels during *in vitro* growth but may be important in *in vivo* infections. It has been reported that in conditions that mimic the host infection such as iron [97] and oxygen limitation [106] or serum [166] and blood [167] the transcriptome of *N. meningitidis* is considerably altered and, as a result, some virulence factors may be over-expressed. Furthermore, several adhesins are subject to antigenic variation and/or phase variation, which allow bacteria to generate a broad and variable repertoire of surface structure that facilitates evasion of immune effectors mechanisms and adaptation to different niche [44].

Several minor adhesins belong to the family of autotransporter adhesin. Among them, *Neisseria* Hia homologue A (NhhA), mediates low levels of adhesion to epithelial cells and to extracellular matrix components as laminin and vitronectin [168]. More recent studies also support its contribution in colonization by preventing phagocytosis and complement attack [169]. Adhesion penetration protein (App), an autotransporter protein with a highly conserved aminoacid sequence, is present in all *Neisseria* species. It has been shown to mediate bacterial interaction to ephitelial cells during the early stages of colonization, before it is autocleaved. At later stages, App autocleavage may allow bacterial detachment, therefore facilitating bacterial spread [170]. Meningococcal serine protease A (MspA) is homologous to App and may also be cleaved and secreted. It is expressed by several but not all virulent *Neisseria* strains and mediates binding to both epithelial and endothelial cells and elicits the production of bactericidal antibodies [171]. The multiple adhesin family (Maf) is a family of glycolipid adhesins, characterised first in the gonococcus, which may play a role in Opa-independent cell invasion [172]. The neisserial adhesin A (NadA) is a subject of this thesis and will be discussed below in details.

Porins. PorA and PorB, the most abundant proteins present in the outer membrane, are beta-barrel proteins which associate in trimers and function by creating pores for the passage of small hydrophilic solutes necessary for bacterial metabolism. PorA proteins are generally present in most meningococcal strains but their expression varies considerably [78, 173]. While not considered adhesins, they interact with numerous human cell and proteins [174], possibly having implication in pathogenesis and generation of an effective immune response. PorA elicits a protective immune response in humans [175, 176], while

the role of PorB in stimulating immune protection is less clear, being immunogenic [177] but poorly accessible for antibodies [178].

Immune evading mechanisms. The ability to escape the elaborate machinery of the human immune system is a key determinant in the virulence of human pathogens. Many factors contribute to the virulence of *N.meningitidis*, involving mechanism to face antimicrobial peptides, reactive nitrogen and oxygen species, complement-mediated killing and, ultimately, the humoral and cellular components of the immune system. Efflux pump have been shown to have a critical contribution to antimicrobial peptide resistance [179]. Enzymes such as catalases (Kat), superoxide dismutase (SodB and SodC), nitrite reductase (AniA) and nitric oxide reductase (NorB) neutralize the toxic effects of neutrophilis and macrophages reactive oxygen and nitrogen species (ROS) [102, 113, 180, 181]. Moreover, *N. meningitidis* uses a variety of mechanisms to survive to the bactericidal action of the complement system [150], involving the capsule, LOS and other factors. One such factor is the fHbp, which factor H (fH), the main inhibitor of the complement alternative pathway. Sequestering fH allows microbes to use this down-regulator to limit complement activation on their surface. Neisserial Heparin-Binding Antigen (NHBA) has been described to bind heparin which may increase bacterial serum resistance due to the potential interactions of heparin with fH [182].

1.5.1 The Neisserial adhesin A (NadA)

The Neisserial adhesin A (NadA) was firstly identified during a bioinformatic analysis of the genome of a virulent *N. meningitidis* B strain for finding novel vaccine candidates [62, 183].

Structure. NadA is a surface-exposed member of the Oligomeric coiled-coil adhesin (Oca) family of bacterial Trimeric Autotransporter adhesins (TAs), such as YadA of *Yersinia* spp. [184-186], UspAs of *Moraxella catarrhalis* [187, 188], Vomp proteins of *Bartonella quintana* [189], BadA of *Bartonella henselae* [190] and HadA of *Haemophilus influenzae* biogroup aegyptius [191]. All the TAs share an obligate trimeric architecture, formed by three identical inter-winded polypeptides. They are typically organized with a variable N-terminal globular portion, called the passenger, projected into the external environment and comprising the binding site(s) for target cell receptors and a conserved C-terminal domain 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. The C-terminal anchor can form a barrel-shaped channel inserted into the outer membrane, which the passenger domain crosses during its delivery to the bacterial surface [192, 193]. NadA, as a member of this sub-group, is predicted to have a similar structure [194, 195], and the protein is characterized by a high degree of biochemical stability to heat, detergent and reduction [196].

Function. It has been shown that the NadA passenger mediates adhesion to human epithelial cells [197], suggesting a key role of NadA in bacterial adhesion to the naso- and oro-pharyngeal epithelia during meningococcal colonization of the human upper respiratory tract. NadA plays also a role in invasion of the mucosal epithelium as it has been demonstrated to mediate invasion into human epithelial cells [198]. Moreover, this protein is unique among TAs, being able to bind and activate macrophages [199] and dendritic cells [200]. As a consequence of this interaction NadA leads to the maturation of

dendritic cells as well as the activation and differentiation of monocytes into macrophages [199, 201]. In this sense NadA has a role in modulating the immune response after the crossing of the epithelial barrier, by targeting immune cells. NadA is capable of inducing strong cellular immune responses [202], bactericidal antibodies in animal models [183, 202-204] and in humans [205] as well as the secretion of pro-inflammatory signals from monocytes and macrophages [199]. Recent studies indicate that part of the molecule can bind human β1 integrins [206] and extracellular chaperone human heat shock protein Hsp90 [207, 208]. These observations suggest NadA plays a multivalent role during the complex process of meningococcal infection which is not limited to bacterial adherence and invasion of mucosal epithelium, but includes translocation across the mucosal layer and release of chemokines by host dendritic cells, monocytes and macrophages recruited at bacterial entry sites.

Classification and molecular epidemiology. The *nadA* gene is an independent genetic unit [203] and is the result of an insertion of foreign DNA in the meningococcal genome. The GC content of the NadA region is lower than the chromosome, which suggests acquisition by horizontal gene transfer and subsequent limited evolution to generate five variants, each of them including a number of subvariants [203, 209, 210]. NadA-1, -2, -3 and -5 occur in invasive strains, whereas NadA-4 has only been found in strains associated with nasopharyngeal carriage.

NadA is a risk factor for the development of meningococcal disease, as it is 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) [210-213]. All together, the *nadA* gene is carried by about 30% of pathogenic isolates collected

from patients in 5 European countries and the US [211-213]. Only 5% of carriage isolates obtained from healthy individuals harbour the gene [210], furthermore, *nadA* is absent from *N. gonorrhoeae* and the commensal *N.lactamica* and *N. cinerea* isolates.

Regulation and expression. The *nadA* gene shows growth phase dependent expression, reaching a maximal level in the stationary phase [141, 203, 214]. As mentioned above the expression of *nadA* is also subject to phase variation, through the presence of a variable length tetranucleotide repeat upstream of its promoter [141, 145]. 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 [141, 145]. However, the major mediator of the phase variable expression of *nadA* is NadR, which binds to two high affinity sites flanking the tetranucleotide repeat. One operator overlaps the -10 region of the promoter and the transcriptional start site, therefore binding of NadR is consistent with its function as a repressor through sterically hindering RNA polymerase to access to the promoter. The other high affinity operator is on the distal upstream side. The DNA bending protein, Integration Host Factor (IHF), also binds on a single binding site that is located between the two high affinity NadR operators. The ability of IHF to bend DNA may facilitate the looping of the DNA of the *nadA* promoter and bring the distal operator near to the core promoter elements, possibly leading to interactions of NadR dimers present on spatially proximal operators in a mechanism similar to the one described for the *lac* operon [215, 216] and purposed for the gonococcal FarR on the *farAB* promoter [138]. The alpha-subunit of RNA polymerase binds to the distal NadR operator and also immediately upstream of the core promoter overlapping the TAAA tract which may function as UP-like elements. A model for *nadA*

regulation has been proposed in which differential distancing between the NadR operators and the contact points of RNA polymerase result in optimal or suboptimal configuration of the protein complexes and, therefore, in efficient or inefficient NadR-mediated repression and cis-enhancement of RNA polymerase activity on the basal promoter strength [141], (Figure 1.7).

The 4HPA molecule, a catabolite of aromatic amino acids which is commonly found in human saliva [147], is able to induce *nadA* expression by alleviating the NadR-mediated repression, suggesting that NadA may be induced in the mucosal niche which is bathed in saliva, during infection [141]. Moreover, anti-NadA antibodies have been found in sera of young children (age 0.2–4.0 years) convalescing after meningococcal disease, suggesting that during invasive human infection NadA is expressed to an immunogenic level which is sufficient to drive a robust humoral response during infection [205].

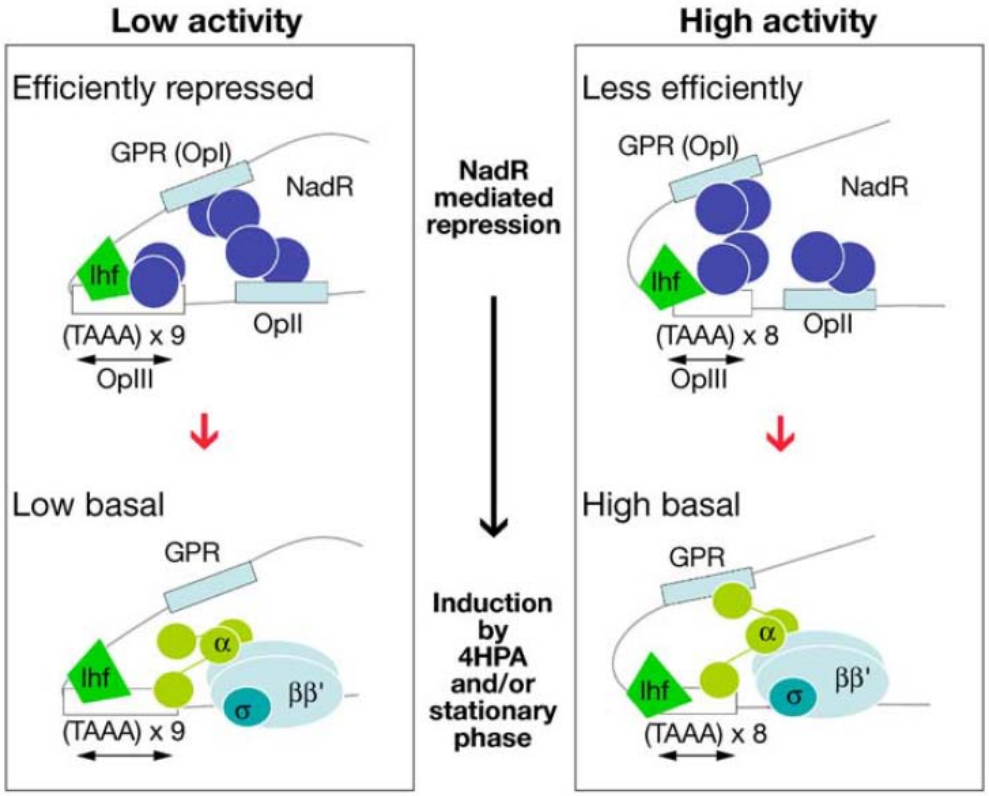


Figure 1.7 - Model of the *nadA* promoter regulation.

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. From [141].

1.6 Meningococcal vaccines

Meningococcal disease progresses rapidly and in its early stages it is easily misdiagnosed [4, 6], making vaccination the best public health option worldwide and the most effective way to prevent it. No broadly protective vaccine is currently available to provide protection against all serogroups of *N. meningitidis*. For four of the five disease-associated serogroups A, C, W and Y, capsular polysaccharides have been successfully used as antigens to produce polysaccharide and glycoconjugate vaccines, which are

currently in use. Quadrivalent vaccines against serogroups A, C, W and Y include the conjugate vaccines Menactra (Sanofi Pasteur) and Menveo (Novartis), and the polysaccharide vaccine Menomune (Sanofi Pasteur), Mencevax (GlaxoSmithKline) and NmVac4-A/C/Y/W-135 (JN-International Medical Corporation) [217]. A vaccine called MenAfriVac has been developed through a program called the Meningitis Vaccine Project to prevent meningitis group A infections in the 'meningitidis belt' [218].

The use of capsular polysaccharide as the basis of a vaccine for prevention of serogroup B strains (MenB) diseases has been problematic. A capsular polysaccharide of MenB is identical to a widely distributed human carbohydrate (α[2-8]*N*-acetylneuraminic acid or polysialic acid), which, being a self-antigen, is a poor immunogen in humans and may elicit autoantibodies [219, 220]. In addition, as mentioned above, the most represented surface exposed protein, PorA and Opa, which should be used as antigens, show sequence and antigenic variability and poor conservation between the diverse strains that cause endemic MenB disease. As a result of these limitations, the only currently available MenB vaccines are the "tailor made" outer membrane vesicle (OMV) vaccines, that have been developed and successfully used to control epidemics that have primarily been caused by a single strain [176]. Although effective, the limitation of the OMV vaccines is that they are strain-specific vaccines and can be used against clonal disease outbreaks but not for prevention of sporadic disease caused by heterologous strains. OMV vaccines have proved to be effective and have been used in Norway [221], Cuba [222], Chile [223] and New Zealand [224] to disrupt homologous strain outbreaks.

1.6.1 Reverse vaccinology and the 4CMenB vaccine

The availability of whole genome sequences in the genomic era has radically changed the approach to vaccine development. To overcome the problems regarding the production of a MenB vaccine, an *in-silico* genome-based approach, called Reverse Vaccinology (Figure 1.8) has been used, allowing the identification of approximately 600 open reading frames that were likely to encode potentially surface exposed proteins in the MenB strain MC58 [183]. The candidate antigens sequences were analysed for their conservation among a wide range of MenB strains and expressed as recombinant protein in *E.coli* to test their surface localization and ability of inducing bactericidal antibodies in mice. This analyses revealed more than 90 previously unknown surface-located proteins, 29 of which were able to induce bactericidal antibodies, the favourites of which were selected for further studies [203, 225-228].

This approach has led to the development of a novel four component recombinant protein vaccine against MenB named 4CMenB [204, 229]. 4CMenB has progressed through clinical trials that have demonstrated its safety [230, 231] and its efficacy in inducing a protective immune response in infants, children, adolescents and adults against potentially the majority of MenB strains [232, 233]. The 4CMenB vaccine contains five Genome-derived *Neisseria* Antigens (GNA), which are formulated as one single recombinant protein, the Neisserial adhesin A (NadA) [198, 203], and two recombinant fusion proteins, the factor H binding protein (fHbp) [234, 235] and Neisserial Heparin-Binding Antigen (NHBA) [182, 226] fused with meningococcal gene products GNA2091 and GNA1030, respectively. NadA, fHbp and NHBA are the major antigens that were selected through reverse vaccinology, based on their ability to induce broad protection

inferred by serum bactericidal activity (SBA) assays or observed in passive protection in the infant rat or mouse protection assays [204]. GNA2091 and GNA1030 are two accessory antigens, well conserved in *N. meningitidis* strains but less well functionally characterized than the major antigens. They are included in 4CMenB formulation as the fusion proteins induce more potent immune responses in SBA than the individual antigen [204]. The vaccine also contains Outer Membrane Vesicles (OMVs) from the meningococcus B strain NZ98/254 in which PorA serosubtype 1.4 represents the major antigen [236].

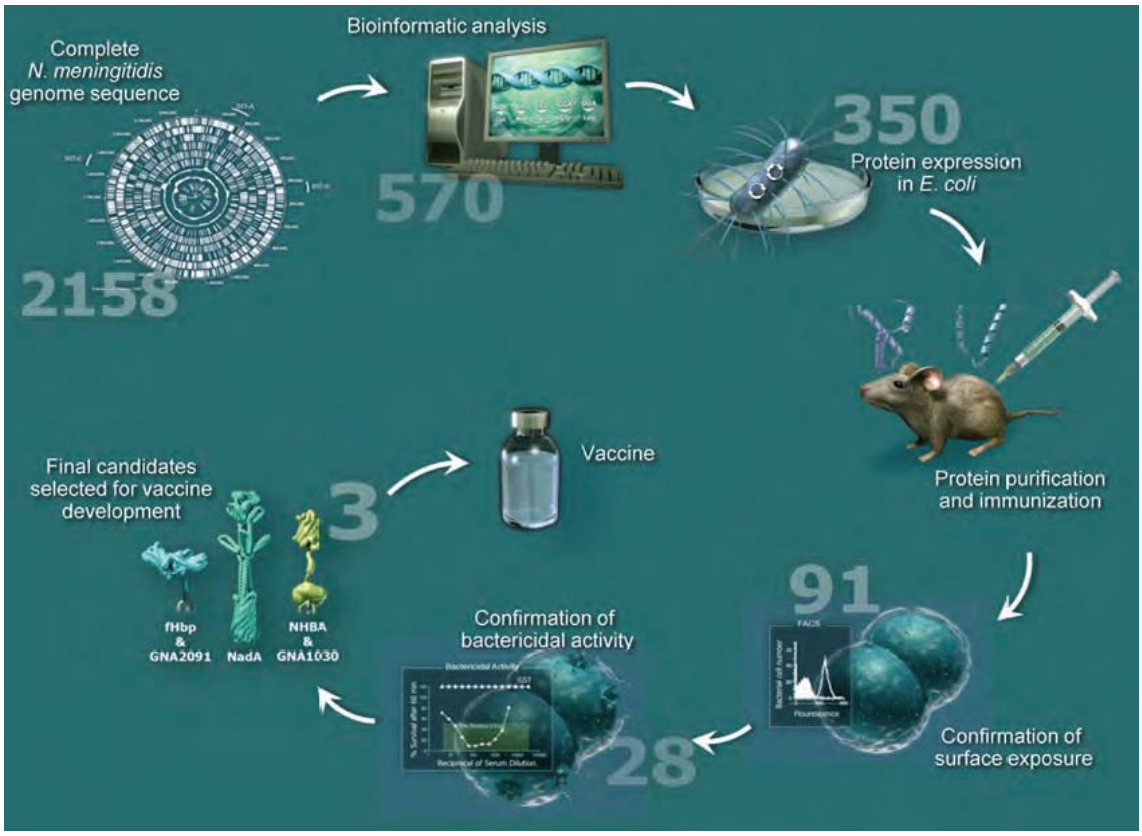


Figure 1.8 - Reverse vaccinology.

The process of reverse vaccinology applied to the development of the 4CMenB vaccine is schematized here and described in details in the text.

1.6.2 Vaccine coverage prediction

The studies performed by Goldschneider in 1969 demonstrated that the presence of bactericidal antibodies in the serum, assessed by serum bactericidal assay with human complement (hSBA), correlates with resistance to meningococcal meningitis. Therefore, hSBA is widely accepted as a surrogate marker of protection against meningococcal disease [12, 237-239]. hSBA assay made possible the development of meningococcal vaccines based initially on the serogroup A, C, Y and W capsular polysaccharides, and later, on these same polysaccharides covalently conjugated to protein carriers [240-242].

However, using hSBA in measuring the potential effectiveness of the 4CMenB vaccine to kill circulating serogroup B strains presents two problems: on one hand, it requires performing the assay against many diverse strains for each geographic region, which is impractical, especially for infants, where serum volumes are very limited, on the other hand the hSBA reflects the cumulative effect of all of the antibodies present in a serum sample in killing the bacteria, making it dificult to understand the contribution of each individual antigen to the function of the four component 4CMenB vaccine.

In order to overcome these issues and predict the coverage of 4CMenB, and a novel assay has been developed, the Meningococcal Antigen Typing System (MATS) [243], (Figure 1.9). MATS assesses simultaneously the antigenic cross-reactivity and the level of expression of the antigens present on the surface of an unknown meningococcal isolate with respect to reference MenB strains for a specific antigen. MATS uses ELISA assays to measure the three 4CMenB vaccine recombinant antigens NadA, fHbp, and NHBA. The output value is the MATS Relative Potency (RP), which correlates with the hSBA assay and may predict whether a strain would be killed due to antibodies elicited by the 4CMenB

vaccine. MATS RP threshold values for complement-mediated killing of MenB by antibodies against NadA, fHbp and NHBA antigens was established and termed the Positive Bactericidal Threshold (PBT), for each specific antigen. Regarding PorA, the MATS determines the PorA subtype by serological analysis or by PCR sequencing and strains that carry the P1.4 PorA subtype are considered killed by vaccine-induced immune sera.

MATS can be easily performed in large panel of strains, making it possible to survey large collections of MenB isolates in order to determine the potential for strain coverage by the 4CMenB vaccine of a target geographic region [244, 245]. Currently MATS is being used by several meningococcal reference laboratories to establish the potential coverage of the 4CMenB vaccine in different countries in Europe, North and South America and Australia. According to MATS, it has been estimated that 78% of circulating MenB strains in five European countries would have at least one antigen rated above the PBT and therefore would be covered by the 4CMenB vaccine. However, the estimated contribution of the NadA antigen to the vaccine coverage would comprise only about 2% of the tested strains [246, 247]. Currently, the amount of the 4CMenB antigens expressed by meningococci during human pathogenesis is unknown, but accurate estimates are important to evaluate the real effectiveness of the vaccine. The study of NadA expression during host infection and its implication on vaccine coverage prediction are a focus of this thesis.

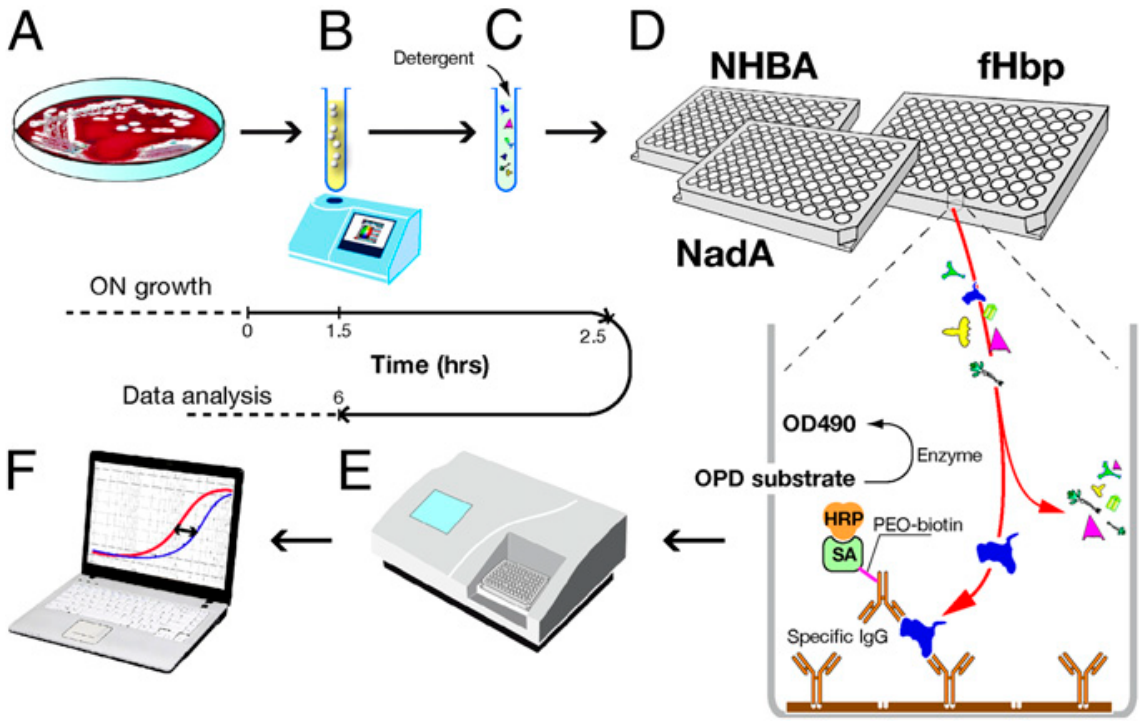


Figure 1.9 - Schematic of the MATS ELISA method.

(A) MenB bacteria are grown overnight on chocolate agar. (B) A suspension of bacteria taken from the plate is prepared to a specified OD600. (C) Detergent is added to the suspension to extract the capsule and expose the antigens. (D) Serial dilutions of extract are tested in the MATS ELISA. A specific capture antibody (yellow) binds one of the antigens (example: fHbp, blue) from the extract, which is then detected with a specific biotin-labeled antibody (yellow and purple) and a streptavidin–enzyme conjugate (green and gold). (E) Plates are read at 490 nm in an ELISA reader. (F) Results are calculated by comparing the curve of OD490 vs. dilution obtained with the serially diluted unknown strain to a serially diluted reference strain tested in the same ELISA plate. From [243].

2 RESULTS

2.1 In the NadR regulon, adhesins and diverse meningococcal functions are regulated in response to signals in human saliva

The meningococcal NadR was shown to repress expression of the NadA adhesin and play a major role in NadA phase variable expression [141]. However, while *nadA* is present in about 30% of circulating strains, NadR is always present and well conserved in all meningococcal genomes available.

The aim of this part of the thesis is to clarify the global role of NadR during menincoccal pathogenesis. We wish to elucidate the NadR regulon by finding its gene targets and the kind of regulation mediated on them. We would also like to understand how the NadR activity is affected by the 4HPA molecule, that is present in saliva and, therefore, in the niche of meningococcal colonization, and which was previously shown to induce *nadA* expression [141].

2.1.1 Global analysis of gene expression in the NadR mutant

In order to identify NadR-regulated genes in *N. meningitidis* we used a custom-made Agilent oligonucleotide microarray to compare the transcriptional profiles of MC58 wild type and MC-Δ1843 NadR mutant strains grown until mid-log phase. Three independent 2-colour microarray experiments were performed comparing pooled RNA from triplicate cultures of each strain, as well as three dye-swap experiments. The results obtained from these experiments were averaged after slide normalization and 28 differentially expressed genes were identified with a log2 ratio >0.9 transcriptional change and a t-test

statistics p-value ≤ 0.01 (Table 2.1.1). When we reduce the criterion to log2 ratio >0.6 in the original 3 experiments (excluding the dye-swap) we can select another 9 differentially up regulated genes in the NadR mutant with a p-value ≤ 0.01 (Table 2.1.1). RT-PCR analyses were consistent with the microarray data (Table 2.1.1) confirming the putative NadR-regulated genes identified in this global gene expression analysis. 31 genes were up-regulated, whereas 6 were down-regulated in the NadR mutant. The contiguous genes that are in the same orientation and exhibiting similar regulation have been grouped into likely operons (Table 2.1.1).

In order to confirm that the regulation is NadR dependent, we complemented the MC-Δ1843 mutant by reintroducing the *nadR* gene under the control of an inducible P_{tac} promoter in a different locus in the genome, generating the strain ΔNadR_C. By altering the concentration of IPTG in the growth medium of the ΔNadR_C strain, we obtained a level of NadR expression in the complemented strain comparable to that of the wildtype (data not shown) and prepared total RNA from this strain in the same condition as the microarrays experiments. We measured the transcriptional levels of the target genes by RT-PCR in the complemented ΔNadR_C strain and observed that for all of the genes tested that were up-regulated in MC-ΔNadR (MC-ΔNadR vs MC58), complementation of the mutant (NadR_C vs MC58) restored their expression to levels similar or slightly lower than the wild type level. In contrast, none of the down-regulated genes was restored to wild type through complementation of the mutant, suggesting that these effects were not dependent on the activity of the NadR protein. Among the 6 down-regulated genes NMB1844, NMB1842 and NMB1841 are contiguous and in the same orientation to the NadR-encoding gene NMB1843 and may be polarly affected by the insertion of the

antibiotic resistance cassette for the generation of the mutant. The lack of complementation of deregulation of NMB0119 and NMB0120 is instead likely to be due to a *nadR*-independent effect such as phase variation or secondary mutations.

These data suggest that NadR functions solely as a repressor protein, negatively regulating multiple loci. Through global gene analysis we identify at least 18 hypothetical operons/transcriptional units coding for 31 genes whose expression was repressed by the *nadR* gene (Figure 2.1.1).

Table 2.1.1. Differentially regulated genes in NadR null mutant

			Microarray results Δ1843 vs MC58wt		RT-PCR Fold change		
Gene Name	**Ori[A]**	**Function**	**Average[B] (Fold Change)**	**Pval**	**Δ1843 vs MC58**	**NadR_C vs Δ1843**	**Gel shift**
NMB0119	↑	hypothetical protein	-4.5	1.8E-14	-2.7	1.1	
NMB0120	|	hypothetical protein	-8.4	2.3E-06	-4.4	1.1	nd
NMB0207	↓	glyceraldehyde 3-phosphate dehydrogenase, *gapA*	4.0	8.5E-07	5.4	-5.9	+
NMB0375	|	MafA1 adhesin, *mafA1*	2.1	4.4E-05	2.3	-2.3	+
NMB0374[D]	↓	MafB1 adhesin, *mafB1*	1.3	4.7E-02	nd	nd	
NMB0401	↓	proline dehydrogenase, *putA*	1.9	1.2E-04	2.4	-3.1	+
NMB0429	|	hypothetical protein, putative regulatory protein	1.9	3.1E-06	nd	nd	+
NMB0430	|	putative carboxyphosphonoenolpyruvate phosphonomutase, *prpB*	1.9	9.6E-04	nd	nd	
NMB0431	↓	methylcitrate synthase/citrate synthase 2, *prpC*	1.9	1.6E-04	nd	nd	
NMB0652	|	MafA2 adhesin, *mafA2*	2.0	1.4E-04	2.3	-2.3	+
NMB0653	|	MafB-related protein, *mafB2*	1.9	6.8E-07	nd	nd	
NMB0654	↓	hypothetical protein	2.1	3.6E-03	nd	nd	
NMB0865	↑	hypothetical protein	1.9	4.0E-05	2.8	-5.5	nd
NMB0866	|	hypothetical protein	2.0	8.9E-03	nd	nd	
NMB0955	|	2-oxoglutarate dehydrogenase, E1 component, *sucA*	1.9	2.7E-07	2.9	-3.2	+
NMB0956[D]	↓	2-oxoglutarate dehydrogenase, *sucB*	1.6	2.7E-02	nd	nd	
NMB1205	↓	hypothetical protein	2.4	8.4E-04	nd	nd	+
NMB1277	↑	transporter, BCCT family	1.9	1.2E-06	1.7	-4.5	+
NMB1299	↓	sodium- and chloride-dependent transporter	2.2	3.2E-03	nd	nd	-
NMB1476	↑	glutamate dehydrogenase, NAD-specific, *gluD*	4.1	2.3E-11	7.9	-11.1	+
NMB1477	|	hypothetical protein, *comA* competence protein	2.3	7.0E-03	nd	nd	

NMB1478	|	phosphoglycolate phosphatase	2.3	3.6E-05	3.7	-2.6	-
NMB1479	↓	regulatory protein RecX	2.2	2.1E-06	nd	nd	
NMB1841	↑	mannose-1-phosphate guanyltransferase-related protein	-8.5	1.1E-16	-3.8	1.0	
NMB1842	|	putative 4-hydroxyphenylacetate 3-hydroxylase	-14.1	0.0E+00	-5.6	0.9	
NMB1843	|	transcriptional regulator	-25.5	8.3E-09	-12.3	nd	+
NMB1844	|	hypothetical protein	-3.0	4.3E-06	-1.6	1.1	-
NMB1994	↓	putative adhesin/invasin, *nadA*	59.6	0.0E+00	97.3	-133	+
			Average[C]	**Pval**			
NMB0535	↓	glucose/galactose transporter, *gluP*	1.9	3.6E-04	nd	nd	-
NMB0702	↓	competence protein ComA	1.8	1.1E-03	1.9	-7.1	-
NMB0978	↑	NAD(P) transhydrogenase, beta subunit, *pntB*	1.7	6.4E-03	4.3	-2.9	
NMB0979*	|	Hypothetical protein	1.6	1.3E-01	nd	nd	
NMB0980*	|	NAD(P) transhydrogenase, alpha subunit, *pntA*	1.6	2.8E-02	1.8	-1.2	+
NMB1609	↑	trans-sulfuration enzyme family protein, *metZ* O-succinylhomoserine sulfhydrolase	1.7	2.2E-06	2.3	-4.0	+
NMB2097	↑	hypothetical protein, pykA pyruvate kinase	1.5	2.0E-04	nd	nd	
NMB2098	|	conserved hypothetical protein	1.6	4.1E-03	nd	nd	
NMB2099	|	conserved hypothetical protein, putative 5-formyltetrahydrofolate cyclo-ligase	1.7	4.7E-03	2.4	-4.3	+

A Ori: orientation. Genes are grouped into likely operons as predicted by their similar orientation and proximity. Vertical solid lines represent the first and following genes in the predicted operon. Arrows pointing up represent the last gene in the operon in the reverse strand of the genome. Arrows pointing down represent the last gene in the operon in the forward strand of the genome.

B Average values of three separate microarray experiments and three dye-swap experiments.

C Average values of three separate microarray experiments.

D Genes with values outside the criteria used are included when contiguous and oriented similarly to upstream or downstream co-regulated genes.

E ND, not determined.

2.1.2 Functional classification of the NadR-regulated genes

Among all NadR-regulated genes (Figure 2.1.1), the NadA adhesin shows the most altered expression profile in the Δ1843 mutant. Interestingly, 2 of the 3 *mafA* (multiple adhesin family A) loci were also repressed by NadR. The NadR-regulated MafA1 and MafA2 (encoded by NMB0375 and NMB0652) are expressed in MC58, while *mafA3* is a pseudogene containing a frameshift. The *maf* loci, similarly to the locus of the Type IV pili, consist of downstream silent cassettes of the expressed genes and are thought to undergo antigenic variation through recombination of the coding sequences with the silent cassettes [71]. The *maf* genes encode a family of variable lipoproteins originally identified as glycolipid-binding proteins in pathogenic *Neisseria* [71, 248, 249], which have been shown to adhere to glycolipid receptors on human cells [250, 251], and thus predicted to be adhesins. While only approximately 40% of circulating meningococcal strains carry the *nadA* gene, which is thought to have been acquired by horizontal gene transfer, all meningococcus strains carry multiple loci expressing the Maf adhesins.

In addition to outer membrane adhesins, NadR is able to repress the expression of a number of genes coding for inner membrane transporters including those involved in transport of sugars (NMB0535, glucose/galactose transporter), compatible solutes (NMB1277, encoding a putative glycine betaine transporter), transporters of unknown substrates (NMB1299, encoding an Na- and Cl-dependent transporter) and even DNA (NMB0702, encoding the ComA protein). NadR also regulates genes involved in energy metabolic pathways including the NMB0401 (*putA* encoding proline dehydrogenase), NMB1476 (*gluD* encoding glutamate dehydrogenase) and NMB0955-957 (*sucAB-lpdA1* encoding 2-oxoglutarate dehydrogenase) genes involved in sequential steps of L-proline

and glutamate catabolism, along with other genes that may be involved in amino acid metabolism (NMB1609, NMB1842), and other energy metabolic processes (NMB0207, *gapA-1* encoding a glyceraldehyde 3-phosphate dehydrogenase; NMB0430-431 coding for a putative 2-methylcitrate pathway; NMB1478, *gph* encoding phosphoglycolate phosphatase; and NMB0978-980, *pntAB* encoding NAD(P) transhydrogenase) as well as a number of hypothetical genes (NMB0865/0866, NMB1477, NMB2099-2097) whose function is unknown. Included also in the list are two possible regulators of gene expression: NMB1479, coding for a putative transcriptional regulator, and NMB1205, which synthesizes a small non-coding regulatory RNA recently named AniS [252].

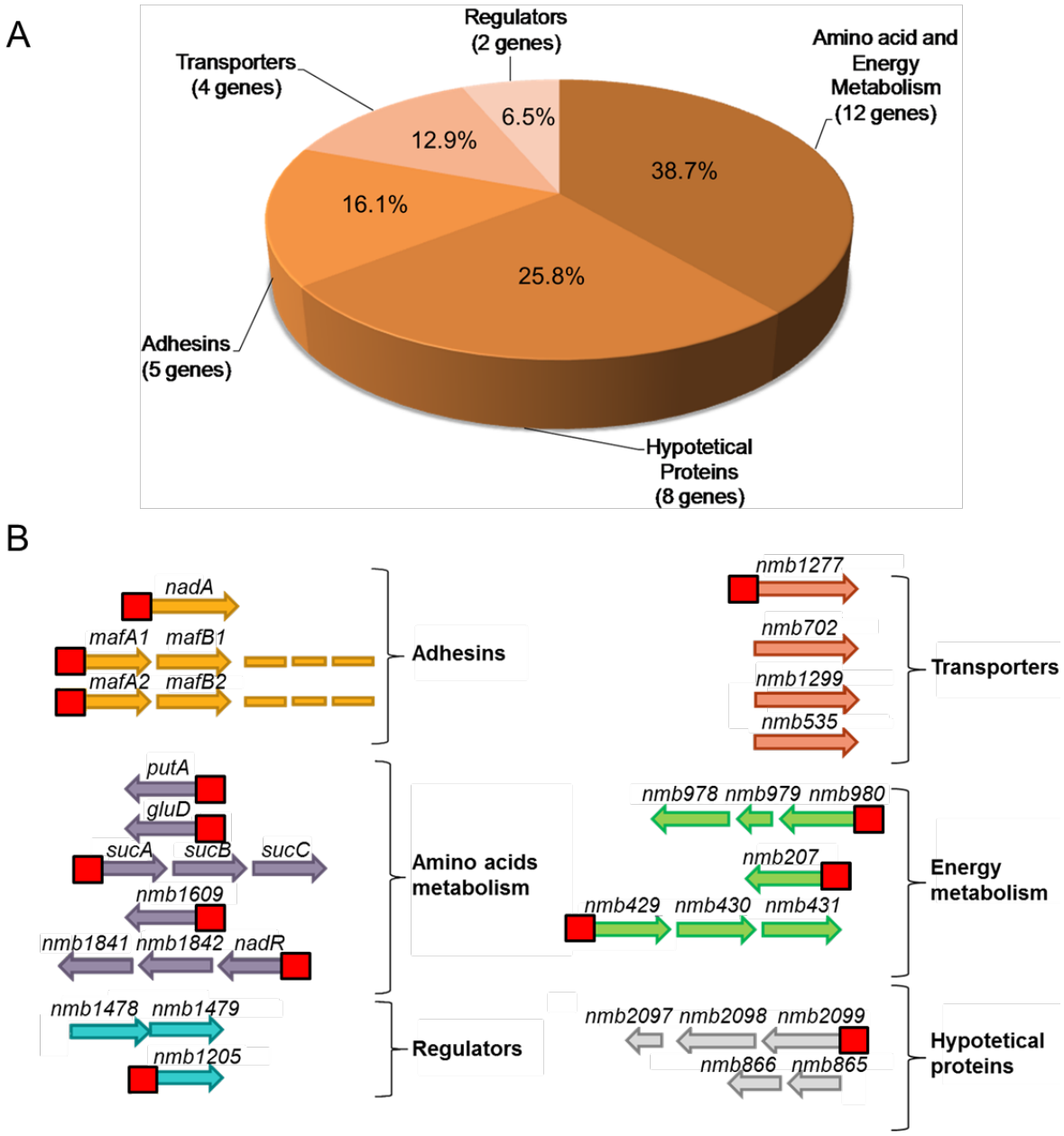


Figure 2.1.1 – Functional classification of the NadR direct and indirect regulated genes.

(A) The diagram shows the numbers of genes in the NadR regulon divided according to their functional classification. Percentages of the total genes in the regulon are reported for each functional class. (B) Schematic representation of the loci controlled by NadR, clustered according to their functional classification. Directly NadR bound targets have been identified through EMSA analyses and indicated by a red box at the 5' of the ORF.

2.1.3 Binding of NadR to its targets

It has been previously demonstrated that NadR binds to the *nadA* and *farAB* promoter regions [141, 253], although no transcriptional regulation of *farAB* is thought to occur in meningococcus [253]. Moreover, it has been recently reported that NadR-regulated genes were not directly bound by the protein [90], suggesting an indirect NadR regulation. To determine which of the NadR-repressed genes are under direct regulation of NadR, we amplified by PCR the upstream promoter regions of identified target genes and performed gel-shift analysis with purified recombinant NadR. We found that out of 19 target promoter regions tested, 14 are bound by the NadR recombinant protein (Table 2.1.1 and Figure 2.1.1B). Interestingly, NadR binds its own promoter, suggesting a possible auto-regulation. The five promoter regions not directly bound by NadR were those of NMB0535, NMB0702, NMB1299, NMB1844 and *gph* (NMB1478), although we cannot exclude that the promoters and therefore NadR regulatory sites are further upstream than the regions tested.

In order to demonstrate the specificity of NadR binding on its targets, a gel shift experiment on the promoter region of NMB0401/*putA* was performed in the presence of increasing concentration of sonicated salmon sperm DNA (non-specific competitor) or cold *putA* promoter (specific competitor) (Figure 2.1.2A).

One slow migrating radioactive complex is formed by adding approx 3 nM of NadR protein to the labeled *putA* promoter. A second complex appears by increasing the concentration of NadR to 12.5 nM. 1-fold of cold *putA* promoter probe is sufficient to abolish the higher complex and 5-fold completely prevents the binding of NadR to the

labeled probe. Instead, up to 25-fold of non-specific competitor had no effect on protein-DNA complex formation, showing that NadR binds specifically to this promoter gene.

A panel of representative gel shift experiments from Table 2.1.1 is shown in Figure 2.1.2B. All these gel shifts were performed in the presence of more than 10-fold excess of non-specific competitor DNA in order to avoid non-specific NadR binding. It is worth noting that with the exception of the promoter regions of NMB0375 (Figure 2.1.2B) and NMB0652 (data not shown) genes, coding for MafA1 and MafA2 respectively, all other promoters exhibit multiple DNA-protein complexes, and therefore are likely to contain multiple binding sites for NadR.

We conclude that NadR can bind to the upstream promoter region *in vitro* of at least 14 transcriptional units consisting of 26 genes, confirming that these genes are members of the NadR regulon. NadR regulation of the other genes may occur through indirect mechanisms such as the regulation of an intermediate regulating factor.

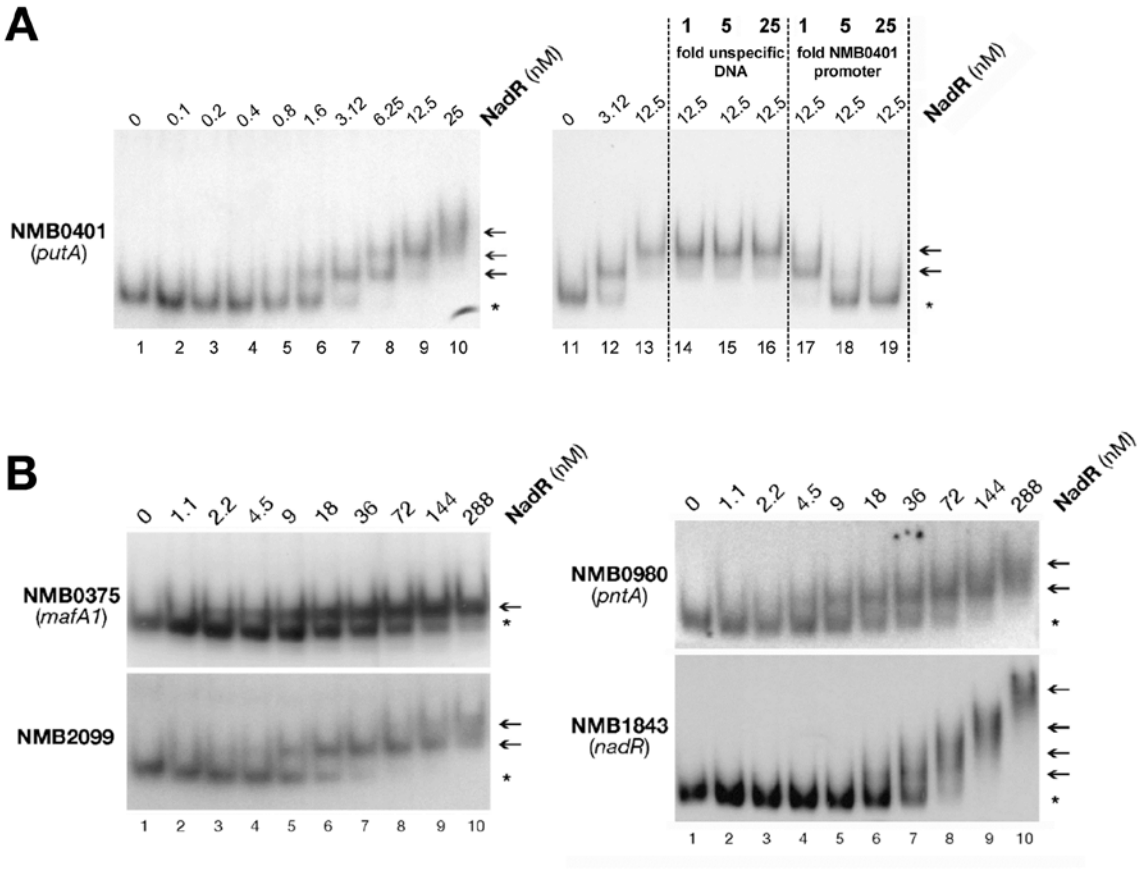


Figure 2.1.2 - NadR binds to its target genes in a specific way.

(A) Gelshift analysis of *NMB0401*/*putA* promoter region. In the left panel 40 fmols of labeled probe was incubated with increasing amounts of NadR protein corresponding to the indicated concentrations. To test the specificity of NadR binding at the *NMB0401* promoter region, in the right panel the same amount of probe was incubated either without or with increasing amount of non-specific (sonicated salmon sperm DNA) or specific competitor DNA (not labeled NMB0401 promoter probe) , in presence of the indicated concentrations of NadR protein. (B) A representative panel of gelshift experiments reported in Table 1 is shown. Promoter regions of indicated targets were amplified by PCR and radioactively end-labeled. 40 fmols of labeled probe was incubated with 2 µg of non-specific competitor DNA and increasing amounts of NadR. Asterisks indicate free probes and arrows indicate DNA-protein complexes.

2.1.4 The NadR target genes can be classified in two types regarding their promoter architecture

The previously described promoter region of *nadA* includes two distally spaced high affinity binding sites for NadR (named OpI and OpII) and one with lower affinity interposing the others (named OpIII) ([141] and schematic representation in Figure 2.1.3B). The presence of multiple NadR binding sites was suggested by gelshift analysis on all NadR identified targets except for NMB0375 and NMB0652. To better understand possible differences between promoter architectures of NadR regulated genes we performed DNase I footprint analysis of NadR purified protein on a representative panel of promoter regions (Figure 2.1.3A).

Both the promoter region of *nadR*/NMB1843 and *putA*/NMB0401 display two distally spaced NadR protected regions, one overlapping the predicted promoter sequences and the second distally upstream. This promoter architecture is comparable to the well-known promoter region of NadA (Figure 2.1.3B) where the OpII overlaps the –10 box of the promoter and the OpI is distally spaced. On the other hand, the promoter region of *mafA1/*NMB0375 (Figure 2.1.3A and schematic representation in Figure 2.1.3B) comprises only one binding site for NadR which overlaps the +1 and downstream of the promoter.

Taken together these data suggest the existence of two types of promoter architecture for NadR regulated genes: type I genes, including *nadA* and the majority of genes of the regulon, have multiple NadR binding sites and in particular two high affinity sites, one overlapping the promoter (centered at -4, -19 and -13 position for *nadA*, *nadR* and *putA*, respectively) and one distally upstream (centered at -129, -103 and -79, respectively);

type II genes, including the *mafA* coding genes NMB0375 and NMB0652, have one single NadR binding site centered at the +7 position of the promoter region.

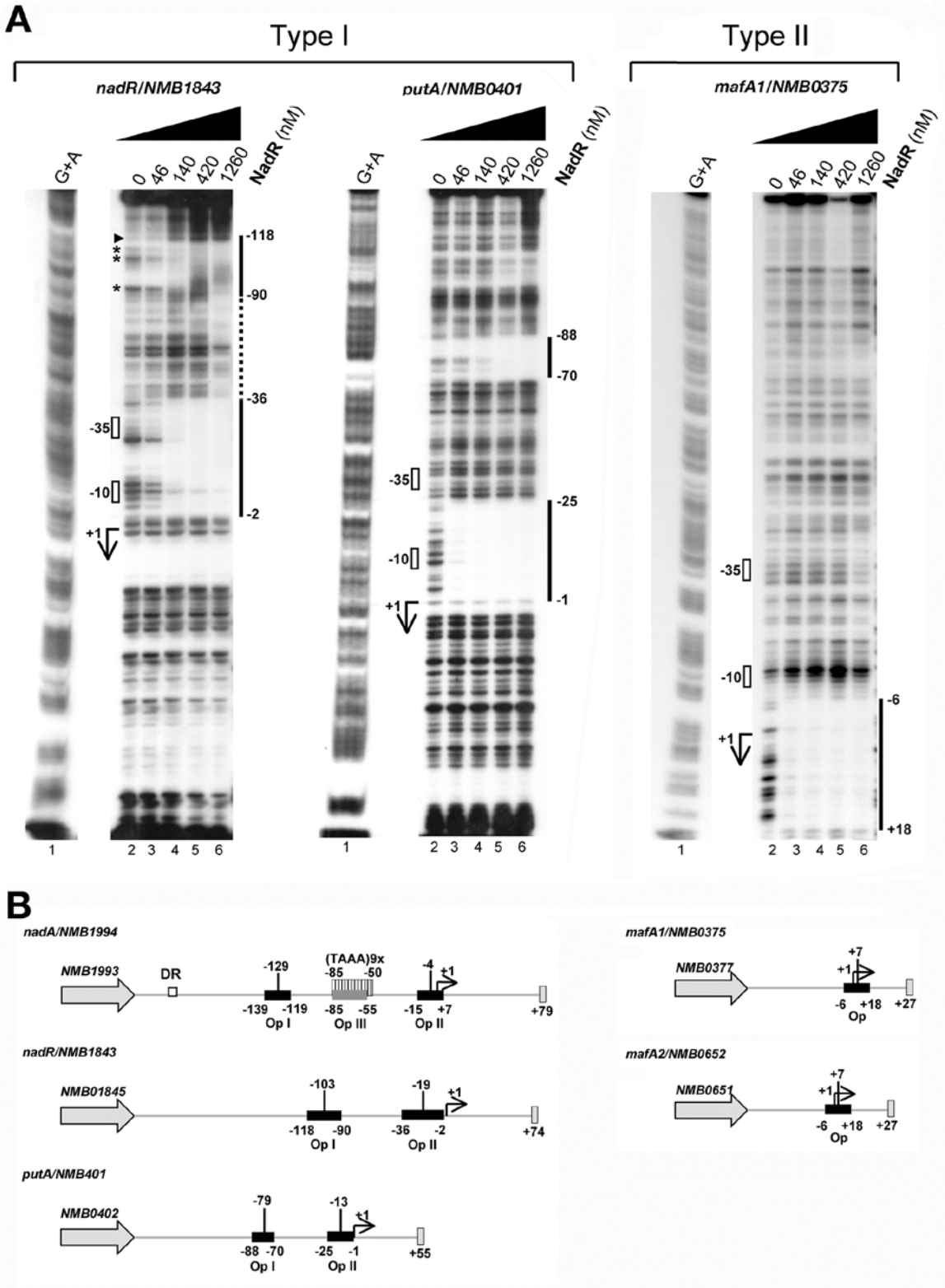


Figure 2.1.3 - Type I and type II genes have different promoter architectures.

(A) DNase I footprint analyses of NadR on a representative panel of the promoter regions of its target genes. 40 fmol of each probe were incubated with increasing amount of NadR purified protein as indicated and then cleaved with DNase I. Regions of protection are indicated with vertical unbroken lines. Regions of non-specific protection, possibly due to multimerization of NadR purified protein, are indicated with vertical dotted lines. Hypersensitive bands are marked by a head arrow; protected disappearing bands are indicated with asterisks. The predicted +1, -10 and -35 of each promoter region were deduced from the DNA sequences and are properly located with respect to the G+A sequences. The positions of NadR protected regions are also indicated. Two classes of promoter architectures are indicated as type I and type II. (B) Schematic representations of type I and type II promoter regions. The structure of the *nadA* promoter is reported as previously described [146], [145] and [141]. The low affinity operator III (Op III) at the TAAA repeats tract is represented by a dark grey box. Regions bound and protected by NadR in DNase I footprint analysis are represented by black boxes. The putative +1 of *putA, nadR mafA1/2* promoters are shown. The centre of each NadR binding site is reported as well. DR, direct repeat (border of region of horizontal transfer).

2.1.5 Ligand-responsive regulation of NadR target genes expression by 4HPA

We have previously shown that a small molecule, 4-hydroxyphenylacetic acid (4HPA), regulates NadR-mediated repression of NadA expression in meningococcus [141]. Therefore, we investigated the 4HPA-responsive regulation of the genes belonging to the NadR regulon. To understand the *in vivo* effect of the small molecule on target gene expression, we quantitatively analyzed the transcriptional level of selected target genes in the MC58 wild type and NadR null mutant in the presence or absence of 5 mM 4HPA, using RT-PCR (Figure 2.1.4). Surprisingly, we found that not all targets respond in a similar fashion to the 4HPA inducer molecule and two classes of gene targets could be defined, corresponding to the two types of genes identified due to their promoter region architectures. We found that the expression of most genes was induced by the 4HPA molecule (type I genes), either partially (i.e. *nadA*) or fully (i.e. *putA, gph, nmb1277)* with respect to the maximal derepression achieved in the NadR mutant. Intriguingly, we found

that only *mafA* genes (type II genes) expression responds in the opposite way to 4HPA, with *mafA* transcription being repressed in the presence of 4HPA and suggesting that this small molecule acts as a co-repressor of NadR at the *mafA1* and *mafA2* promoters. In the NadR mutant strain 4HPA-dependent regulation is largely absent indicating that NadR is the mediator of the 4HPA transcriptional responses.

This analysis suggests that while NadR represses all genes in its regulon, 4HPA may act as an inducer (type I genes) or a co-repressor (type II genes), resulting in alternative NadR-mediated responses *in vivo*, which can be at least partially due to the differential promoter architectures of the two types of NadR targets.

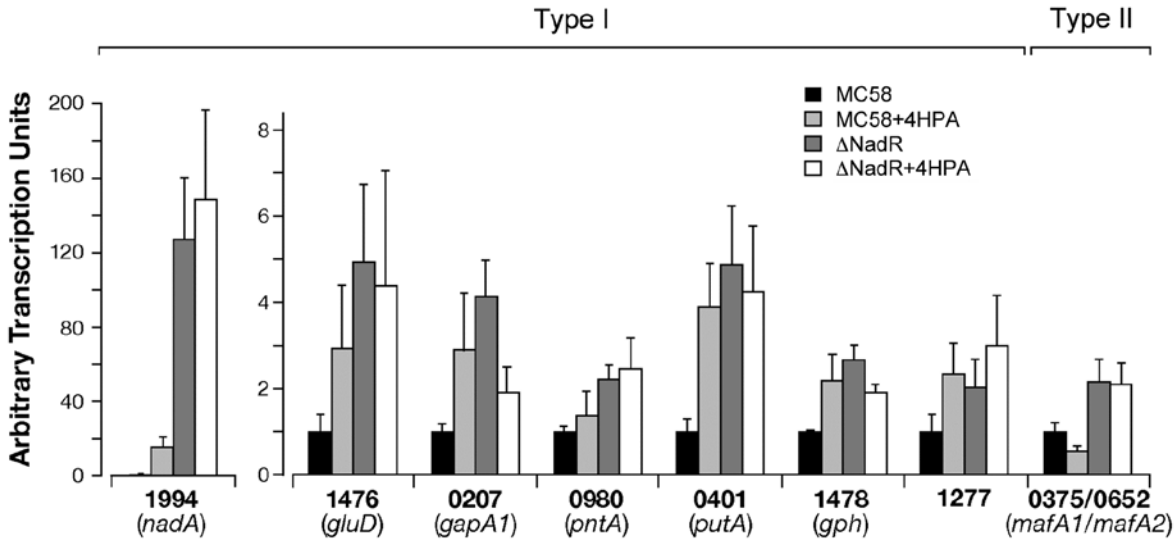


Figure 2.1.4 - Effect of 4HPA on gene expression.

RT-PCR analysis of NadR target transcripts in total RNA prepared from the wildtype (MC58) and MC-ΔNadR mutant strains (ΔNadR) after growth in the presence or absence of 5 mM 4HPA. RT-PCR was performed in duplicate on three independent biological replicates and the average values are shown. The primers used in qRT-PCR for *mafA1* and *mafA2* genes cannot distinguish between the *NMB0375* and *NMB0652* genes as the sequences are identical. Two types of transcriptional patterns can be detected, type I (4HPA-induced) and type II (4HPA-repressed).

2.1.6 The NadR-dependent regulation of NadA, MafA and NadR itself is common among meningococcal strains

In order to investigate whether NadR-dependent regulation of NadA and MafA protein expression was exhibited by other strains, we extended our studies to a larger panel of meningococcal strains. Western blot analysis were carried out on the wild type and their respective NadR mutant strains grown in the presence or absence of 4HPA (Figure 2.1.5). Expression of NadA and MafA is variable between strains, while NadR is expressed to essentially the same level (lanes 1). All NadR null mutants exhibited higher levels of NadA and MafA than their respective wild types (lanes 3 versus 1) confirming the NadR repression of both *nadA* and *mafA* genes. In all the wild type strains, the 4HPA molecule results in induction of both NadA and, to a lesser extent, NadR and co-repression of MafA (lanes 2 versus 1), while it had no effect on NadA or MafA expression in the NadR mutants (lanes 4 versus 3), indicating that the effects of 4HPA are NadR-dependent.

Taken together, these results suggest that both the NadR-dependent regulation of NadA and MafA and the 4HPA activity as an inducer of *nadA* and a co-repressor of *mafA* are not restricted to the MC58 strain but are common throughout different meningococci. Furthermore, it would appear that the level of NadR itself is induced by 4HPA, most probably through alleviation of autoregulatory repression of its own promoter.

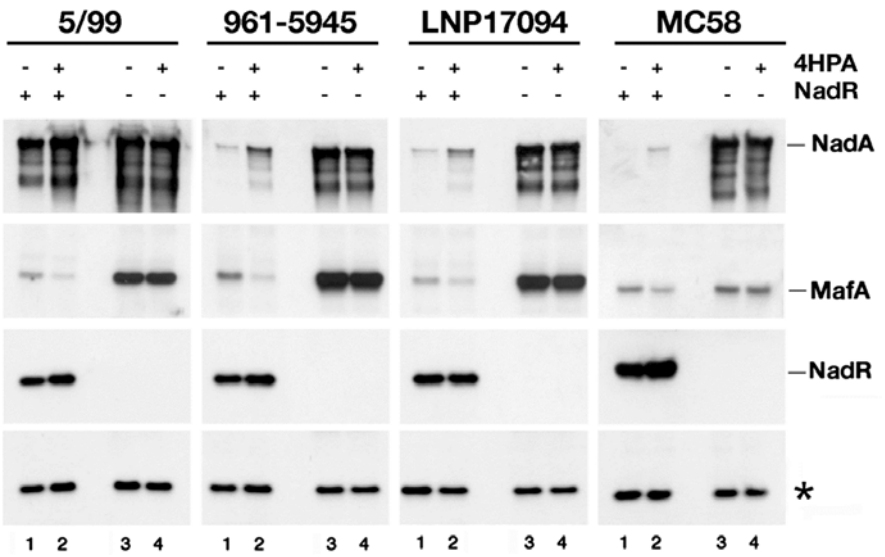


Figure 2.1.5 - NadR-regulated adhesins responses in a broad panel of meningococcal strains.
Western blot of wildtype (lanes 1 and 2) and corresponding NadR knockout (lanes 3 and 4) cultures of the indicated strains grown in the presence (lanes 2 and 4) and absence (lanes 1 and 3) of 1 mM 4HPA as indicated, showing NadA, MafA and NadR expression. The NadA protein migrated at a molecular weight of 98 kDa and corresponds to the trimeric form of the protein [203] and the MafA band migrates at 36 kDa and corresponds to both MafA1 and MafA2 proteins coded for by the *NMB0375* and *NMB0652* genes whose sequences are 100 % identical. This band is absent in Western blots of extracts from the NMB0375 and NMB0652 double mutant [254] (data not shown). A non-specific band indicated with an asterisk is reported as a loading control.

2.1.7 Incubation with human saliva has the same effect on NadA and MafA expression as 4HPA

It has been previously demonstrated that the 4HPA molecule is a catabolite of aromatic amino acids secreted in human saliva [147]. To test whether the effects due to 4HPA *in vitro* may have biological relevance we tried to achieve similar results with human saliva. Mid log cultures of MC58 were incubated for 1 hour either with 4HPA or with increasing amount of human saliva from three different donors. Total protein extracts were obtained and the expression levels of NadA and MafA were analyzed by Western blot (Figure 2.1.6A). The Western blots show the induction of NadA and the repression of

MafA following incubation with 4HPA (compare lanes 2 to 1 of each blot) and, even if with much greater experimental variability, the same results are obtained following incubation with increasing concentration of human saliva from three donors (lanes 3,4,5 compared to 1).

Figure 2.1.6B shows a histogram with average values of quantified signals of Western blot bands from six independent experiments. While NadA is induced by 4HPA as well as by 90% human saliva, MafA expression is co-repressed by both 4HPA and saliva at 50% and 90% concentrations, in a statistically relevant manner with respect to its basal level in GC growth.

This analysis suggests that the 4HPA molecule used in our experiments mimics a signal present in the human saliva, which produces the same regulatory effects on *nadA* and *mafA* expression.

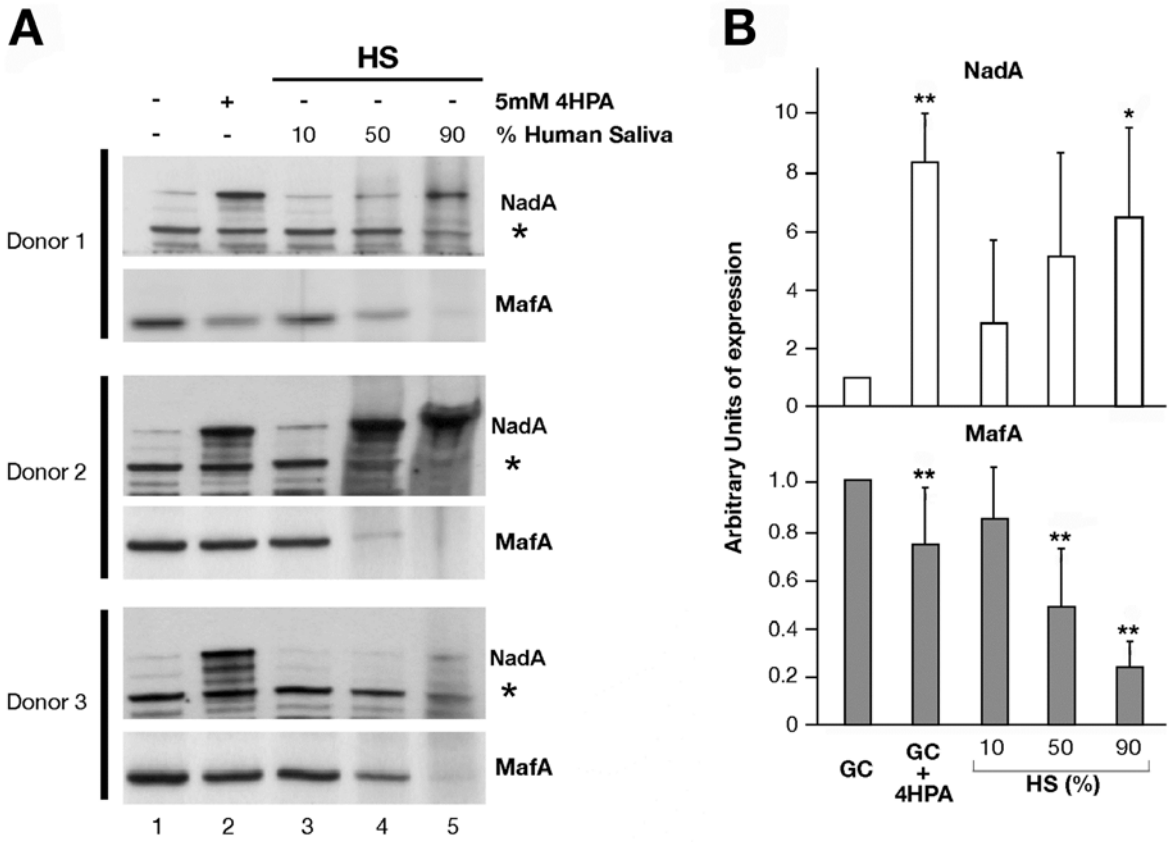


Figure 2.1.6 - Human saliva has the same activity of 4HPA on NadA and MafA expression.

(A) Western Blot analyses of total protein of mid log cultures of MC58 incubated for 1 hour either with 4HPA (lane 1) or with increasing amount of human saliva (HS) from 3 different donors (lanes 3, 4 and 5), as indicated. The levels of NadA and MafA proteins are shown. (B) Histogram representing the average expression levels of NadA (above) and MafA (below). The average values were calculated by quantification of Western Blots bands signals of 2 independent experiments with human saliva of each donor (6 experiments in total). Expression levels of NadA and MafA of cultures grown solely in GC medium were set to 1 arbitrary unit. To note that for the quantification, Western Blots bands were normalized for a non-specific band, indicated with an asterisk in the panel A, in order to avoid non 4HPA- or NadR-dependent effects due to possible protein degradation in saliva. One asterisk (*) means p value < 0.05, two asterisks (**) p value < 0.01, both versus the growth in GC.

2.1.8 4HPA has differential activity on NadR binding to type I and type II promoters *in vitro*

It was previously reported that the 4HPA molecule can attenuate the binding of NadR to the *nadA* promoter *in vitro* [141], supporting the idea that it interacts with the protein

and alters its DNA-binding activity. To investigate the role of 4HPA on the *in vitro* activity of NadR in binding to both type I and type II promoters, we performed gelshift analysis in the presence of NadR and increasing amounts of 4HPA on a selected panel of target promoter regions (Figure 2.1.7A).We demonstrate that for type I (*gap-A1*, *gluD*, *putA, nadR*) promoters, 4HPA is able to inhibit the NadR-DNA binding *in vitro* in a dose-dependent manner , similarly to what we reported for *nadA* [141]. However, addition of 4HPA had no significant effect on binding of NadR at the type II (*mafA1* and *mafA2)* promoters. In a parallel and complementary set of experiments we performed gelshift analysis on the promoters of *nadA* and *mafA1* with increasing amount of NadR purified protein in the presence or absence of 5 mM 4HPA (Figure 2.1.7B-C). While 4HPA has no effect on NadR binding activity on the promoter of *mafA1* (Figure 2.1.7C), in order to have the same amount of DNA-protein complexes on *nadA* promoter, more NadR is required in presence of 4HPA (Figure 2.1.7B), indicating that 4HPA alleviates the binding of NadR on *nadA*. The same results regarding the 4HPA responsive activity of NadR were also observed on distinct NadR binding sites of the *nadA* and *mafA* promoters, as well as using crude extracts from MC58 strain instead of the recombinant purified NadR protein, to ensure that any eventual unknown factor required for NadR mediated repression could be present (data not shown).

This analysis demonstrate that 4HPA affects and decreases the NadR DNA-binding affinity only to type I promoters that are induced by 4HPA *in vivo*, while not having any *in vitro* effect on NadR binding activity on type II promoters that are co-repressed *in vivo*.

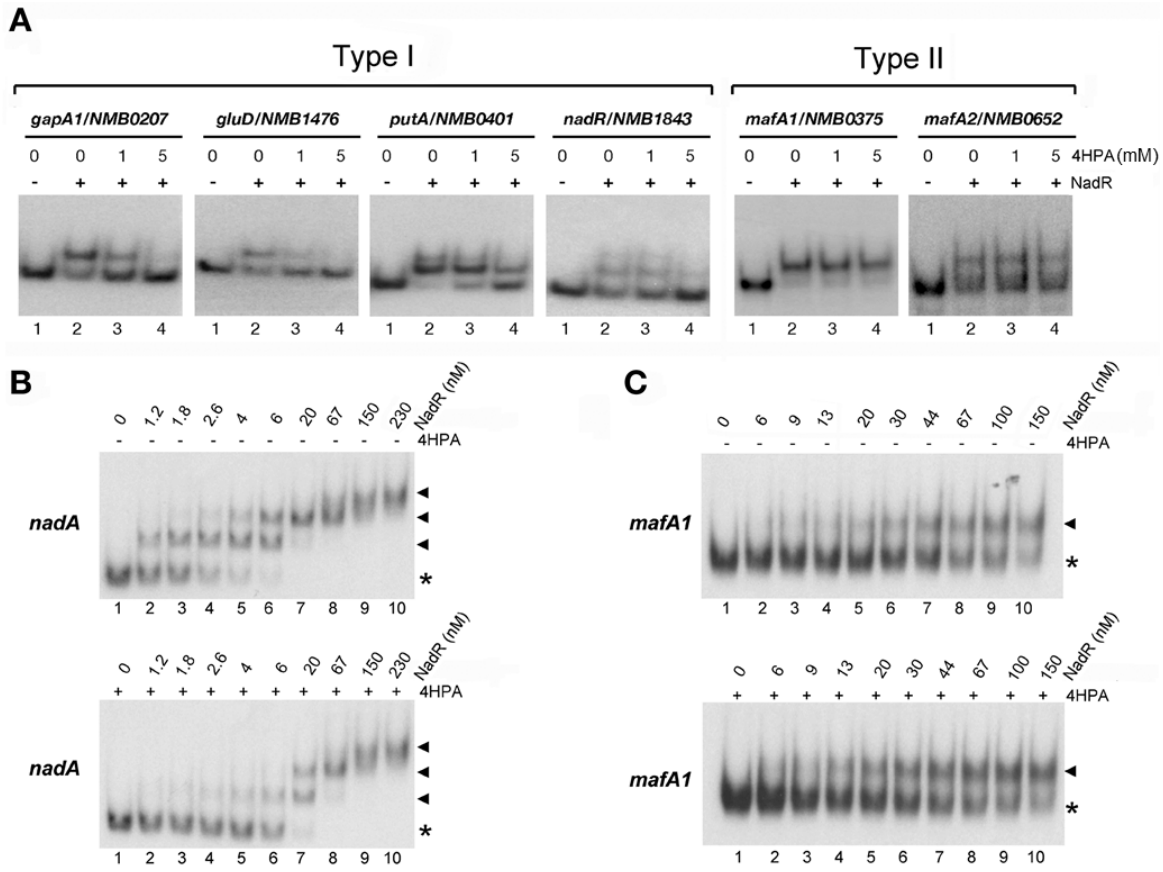


Figure 2.1.7 - NadR responds differentially to 4HPA on type I and type II promoter regions.

(A) Gelshift analysis of selected NadR targets in the presence of increasing concentrations of 4HPA. Radio-actively labeled promoter regions of the indicated genes were incubated with 0 (lane 1) or 36 nM NadR (lanes 2-4) and 4HPA was added at final concentration of 1 mM (lanes 3) and 5 mM (lanes 4). (B) *In vitro* NadR response to 4HPA on the promoter region of *nadA* and (C) *mafA1* studied by gelshift. The *nadA* and *mafA1* promoter regions where amplified with primers pairs Nad-N1/Nad-B1 and P375-F/P375-652R, respectively. 40fmol of end labelled probe were incubated with increasing amount of NadR protein (nM), either with or without 5 mM 4HPA, as indicated. Free probes are indicated by asterisks and DNA bound by NadR is indicated by arrow heads.

2.1.9 The 4HPA mediated co-repression of *mafA* is not due to repositioning of NadR on the promoter

In order to understand the mechanism by which 4HPA mediates the NadR co-repression of the *mafA* promoter, we evaluate the possibility that the 4HPA binding to NadR could cause a conformational change, leading the repressor to bind a different DNA sequence than the one previously observed, causing a more efficient repression. Ligand-induced repositioning of the DNA binding domain is not new and has been described for the MarR-family of transcriptional repressors [255].

To asses this possibility we performed DNAse I footprint analyses of NadR on the *mafA* promoter in absence and in presence of 4HPA (Figure 2.1.8). Ligand binding does not alter the operator bound by NadR on the promoter of *mafA*. Moreover, in agreement with EMSA analyses, the NadR DNA binding activity on the promoter of *mafA* is not altered by 4HPA.

Taken together with the previous data, this indicates that the differential 4HPA responsive activity of NadR on different types of targets is not due to the intrinsic binding activity of the protein and that the nature of the binding site at the type II promoters may be different to those of the type I promoters and these differences may define the regulation to which they will be subjected.

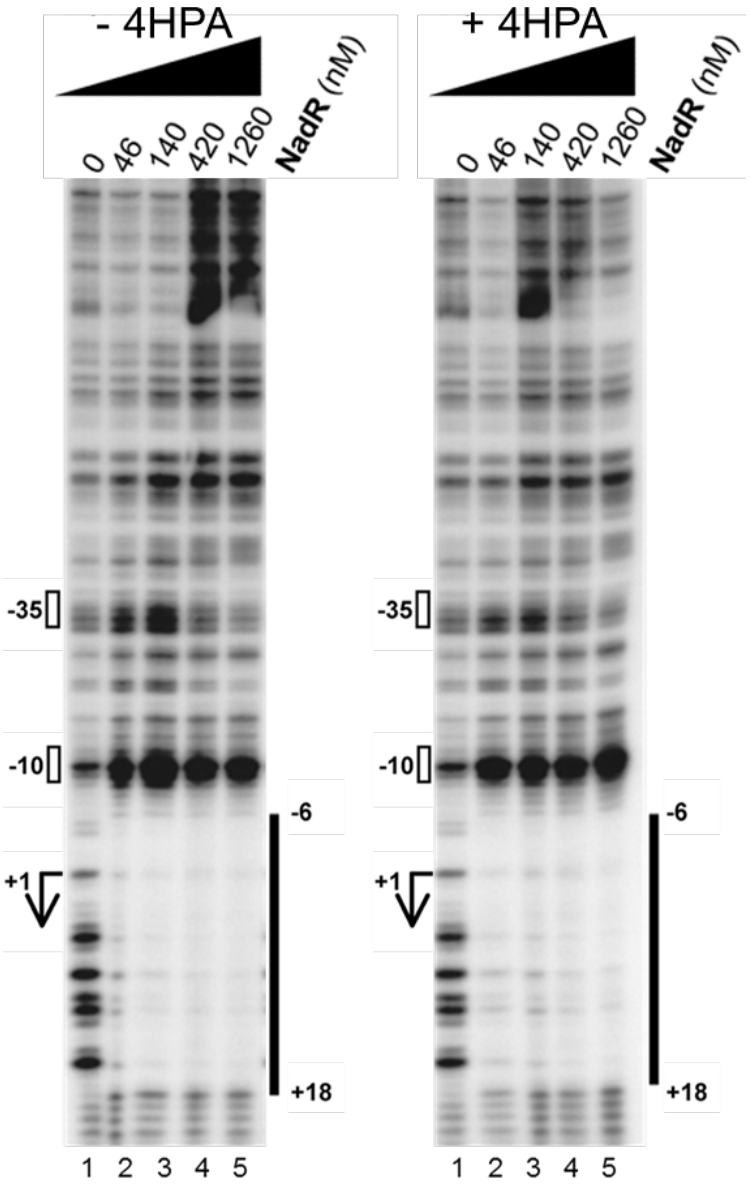


Figure 2.1.8 – 4HPA does not alter the positioning of NadR on the promoter of *mafA*.

DNase I footprint analyses of NadR on the promoter region of *mafA*. 40 fmol of the probe were incubated with increasing amount of NadR purified protein as indicated and then cleaved with DNase I, either in absence (left) or presence (right) of 5 mM 4HPA. Unchanged regions of protection are indicated with vertical unbroken lines. The predicted +1, -10 and -35 are indicated.

2.1.10 3C scanning mutagenesis reveals extended NadR binding sequence in the operator of *mafA* promoter region

In order to elucidate intrinsic differences between single NadR operators at the *nadA* and *mafA1* promoters and the role of nucleotides on NadR binding activity, we generated DNA probes corresponding to the sequences of the high affinity binding sites of the *nadA* (Op I and OpII) and *mafA1* promoters, identified by DNase I footprint. For each probe we designed a set of mutants, in which three nucleotides were sequentially substituted with CCC. To detect the results of the 3C scanning mutagenesis on NadR binding activity, the probes were submitted to EMSA analysis (figure 2.1.9A). By identifying the residues whose mutations do not alter the binding of NadR, we defined the minimal binding site (MBS) required for NadR binding on each operator (figure 2.1.9B). Mutation of nucleotide triplets within the MBS results in either reduced (dark grey residues) or absent (black residues) NadR binding activity. Interestingly, the definition of MBS clearly shows that the operator on the promoter of *mafA1* is significantly extended with respect to the high affinity binding sites in promoter of *nadA*.

These observations suggest a differential mode of NadR binding between the *nadA* and *mafA1* operator, which could be partially responsible for the alternative 4HPA responsive activity of NadR at the two promoters.

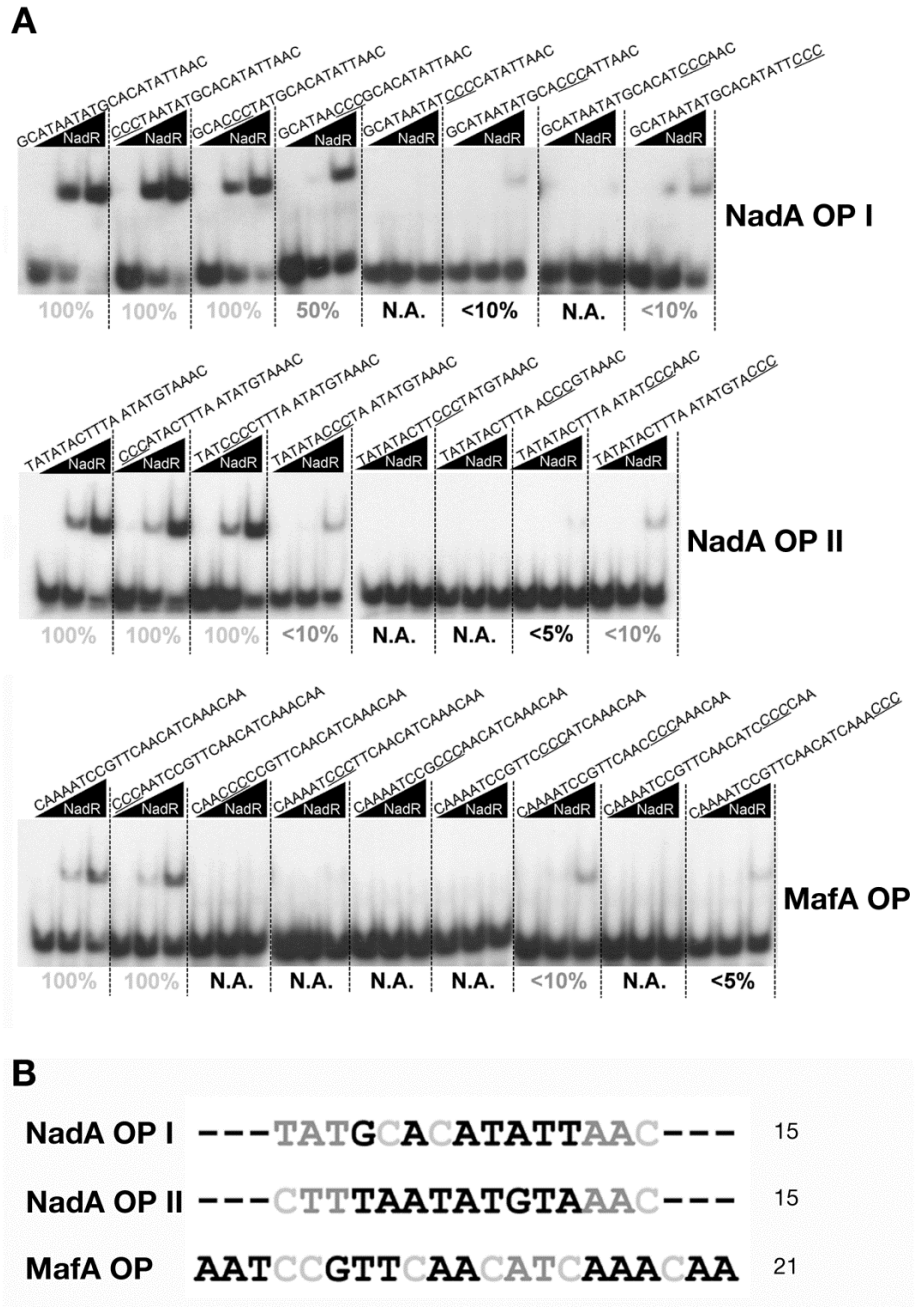


Figure 2.1.9 - 3C scanning mutagenesis of *nadA* and *mafA* single binding sites.
(A) DNA probes corresponding to the wild type NadR-protected sequences from DNAI footprints or to mutated sequences, in which sequential triplicate nucleotides were substituted with CCC, were submitted to gelshift analysis with increasing amount of NadR purified protein (0, 36 and 360 nM, respectively). The mutated nucleotides are in italics and underlined in the sequences above each gelshift. The NadR binding activity to each probe is reported below each experiment as a percentage of the binding activity shown by NadR on the wild type sequence. Black values are associated with mutation causing loss or highly compromised NadR binding activity; dark grey values with less affected NadR binding activity; light grey values with not affected NadR binding activity. (N.A.= not active in binding). (B) The minimal binding sequence (MBS) of each binding site is shown, with nucleotide substitutions essential for binding in black, affecting affinity in dark grey and with no effect in NadR binding activity in light grey.

2.2 Structural insight into the mechanism of DNA-binding attenuation of NadR by the small natural ligand 4HPA

NadR is a member of the MarR family of transcriptional regulators. A characteristic feature of these proteins is their capability to bind and respond to a variety of effector molecules [124, 125]. Although the crystal structures of several MarR homologs have been solved in their apo- and ligand-bound states, the molecular mechanisms of regulation associated with ligand binding is still not known, partly due to the fact that the natural ligand is often not known.

As seen above, 4HPA is a natural ligand of NadR and is a key factor which determines NadR activity. The aim of this part of the thesis is to elucidate the mechanism of regulation of NadR by the 4-HPA molecule. We focused on NadR activity in response to 4HPA on *nadA*, being the gene majorly affected by NadR. We would like to identify the 4HPA binding pocket of NadR and the key residues involved in this domain.

2.2.1 Characterization of the structural model of NadR.

A structural model of NadR was generated using the crystal structure of the transcriptional regulator PA4135 from *Pseudomonas aeruginosa [256]* which shares 42% sequence identities with NadR (Figure 2.2.1A). According to this model, the NadR dimer adopts the typical "triangle" shape of the MarR family with each monomer consisted of the secondary structure elements α1-α2-α3-α4-β1-wing-β2-α5-α6 (Figure 2.2.1B). The two monomers associate *via* a dimerization interface created by their N- and C-terminal helices (α1, α5 and α6) and each monomer contains a winged helix-turn-helix (w-HTH) DNA-binding motif (α3, α4, β1, wing, β2).

The quality of the model was assessed by amide hydrogen/deuterium exchange mass spectrometry (HDX-MS), as reported by Brier and collegues [8]. The rate at which backbone amide hydrogens (NHs) exchange in solution is directly dependent on the dynamics and the structure of the protein [257]. Therefore, regions with secondary structures or occluded from the deuterated buffer will exchange more slowly than regions without any secondary structures and/or fully exposed to the solvent. As shown by data reported in [8] the core of the protein, including the dimerization interface, is well protected from exchange, to generate the well-packed hydrophobic core of the dimerization domain. In contrast, the w-HTH DNA-binding motif was found to be highly accessible, consistent with the fact that these regions must remain fully accessible and/or dynamic to interact with DNA [132]. Altogether, the HDX data correlate well with the homology-based structural model of NadR.

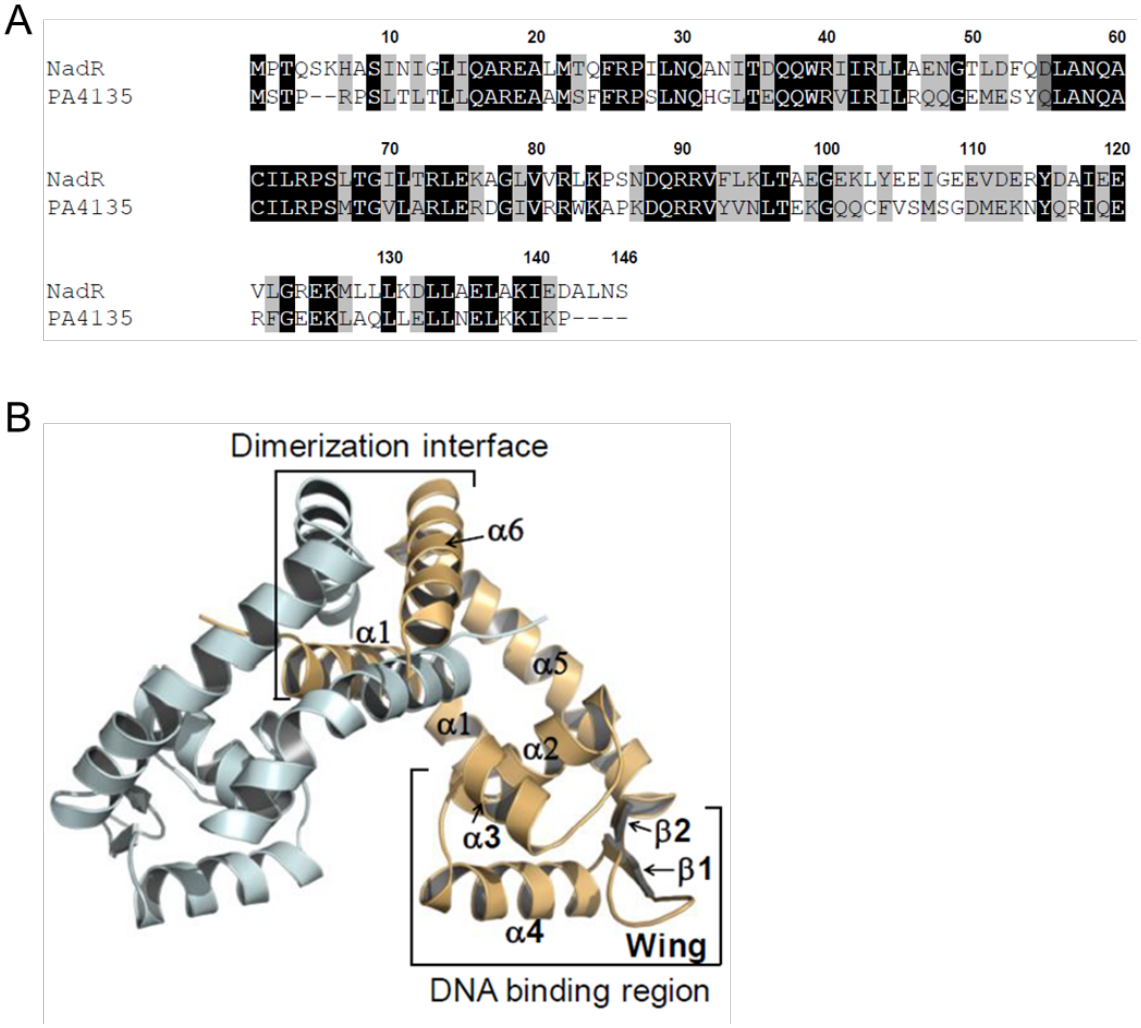


Figure 2.2.1 - Homology model and dynamic analysis of NadR.
(A) Sequence alignment between NadR and transcriptional regulator PA4135 from *Pseudomonas aeruginosa*. NadR shares 42% sequence identity with PA4135. Dark and grey shading indicates the position of the conserved and highly similar residues, respectively. The alignment was performed with CLUTALW. (B) Ribbon representation of the proposed homodimeric structure of NadR obtained with SWISS-MODEL and based on the X-ray structure of PA4135. One subunit is colored in gold and the other in light blue. According to this model, each monomer adopts the typical fold of the MarR family members, which consists of 6 α-helices and 2-stranded β-sheets. The dimerization interface and the DNA-binding regions are indicated.

2.2.2 Localization of the 4HPA binding pocket

To localize the 4-HPA biding pocket, the exchange behaviors of the apo- and HPA-bound forms were monitored and compared by HDX-MS, as reported in [8], resulting in four regions, including Met_{1}-Leu_{14} (N-term α1), Met_{22}-Leu_{29} (C-term α1), Asn_{54}-Cys_{61} (α3) and

Tyr104-Glu119 (α5), protected from exchange upon 4-HPA binding. To visualize more precisely our results, these regions were mapped onto the structural model of the dimer. Interestingly, with the exception of α3, the reduction of solvent accessibility was clustered at the interface between the dimerization and the w-HTH DNA-binding domains (Figure 2.2.2). This interface has already been identified as a common salicylate-binding pocket in several MarR homologues including MTH313 [133], ST1710 [132], SlyA [258] and TcaR [130]. Although salicylate does not bind NadR, based on these observations, we conclude that 4-HPA likely shares the same protein binding region.

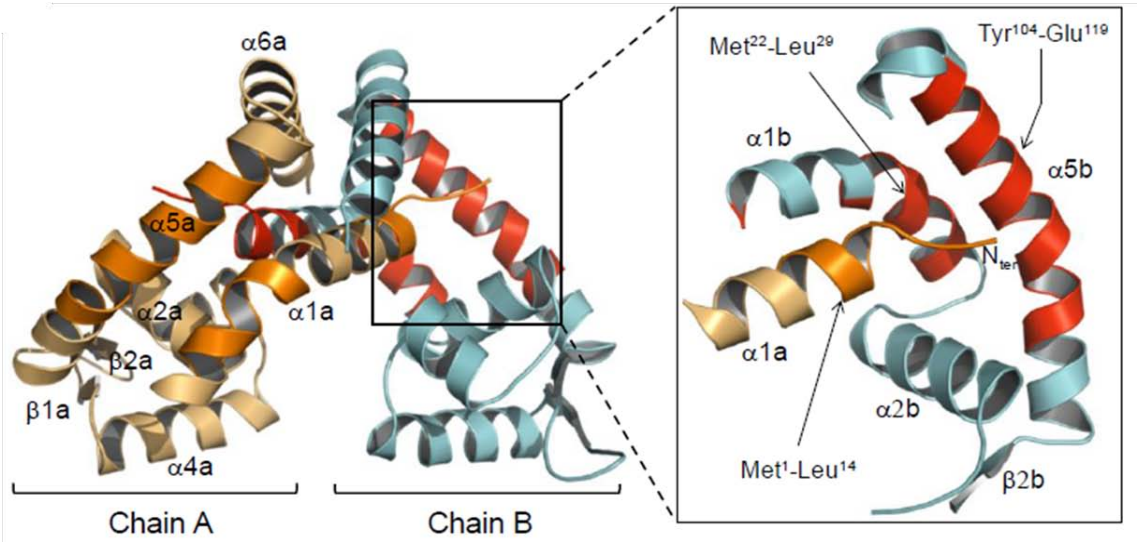


Figure 2.2.2 - The 4HPA binding pocket within NadR.
Ribbon representation of NadR showing the position of the putative 4-HPA binding region identified by HDX-MS. Regions with altered deuterium uptake are colored in orange (chain A) and red (chain B). The 4-HPA-binding pocket is located at the interface between the dimerization and the DNA-binding domains and is mainly formed by the α1 N-terminal domain of chain A and the helices α2, α5 and the α1 C-terminal domain of chain B (see expanded view).

2.2.3 Mutation of key residues in the 4-HPA binding pocket of NadR

In an attempt to identify the residues of NadR in contact with 4-HPA in the identified binding pocket, an *in silico* docking approach has been used as described by Brier and collegues [8]. As a result of this analyses, a predicted network of hydrogen bonds coordinating 4-HPA was observed, which mainly involves residues Arg_{18}, Trp_{39}, Arg_{40}, Arg_{43} and Tyr_{115} (Figure 2.2.3). These residues were selected for mutagenesis to further determine their *in vivo* and *in vitro* functions in 4-HPA binding. Additional residues (His_{7}, Ser_{9}, Asn_{11} and Phe_{25}) were identified through the *in silico* docking approach, and were also mutagenised to assess whether they play a role in the binding of the small molecule. In order to obtain meningococcal strains expressing the NadR mutated forms, the previously described MC58-Δ1843 *nadR* null mutant strain [141] was complemented either by the wild type NadR protein or by the introduction of the desired alanine substituted forms of NadR. Note that we failed to introduce the R43A substitution in NadR and, as a consequence, this residue was not further studied.

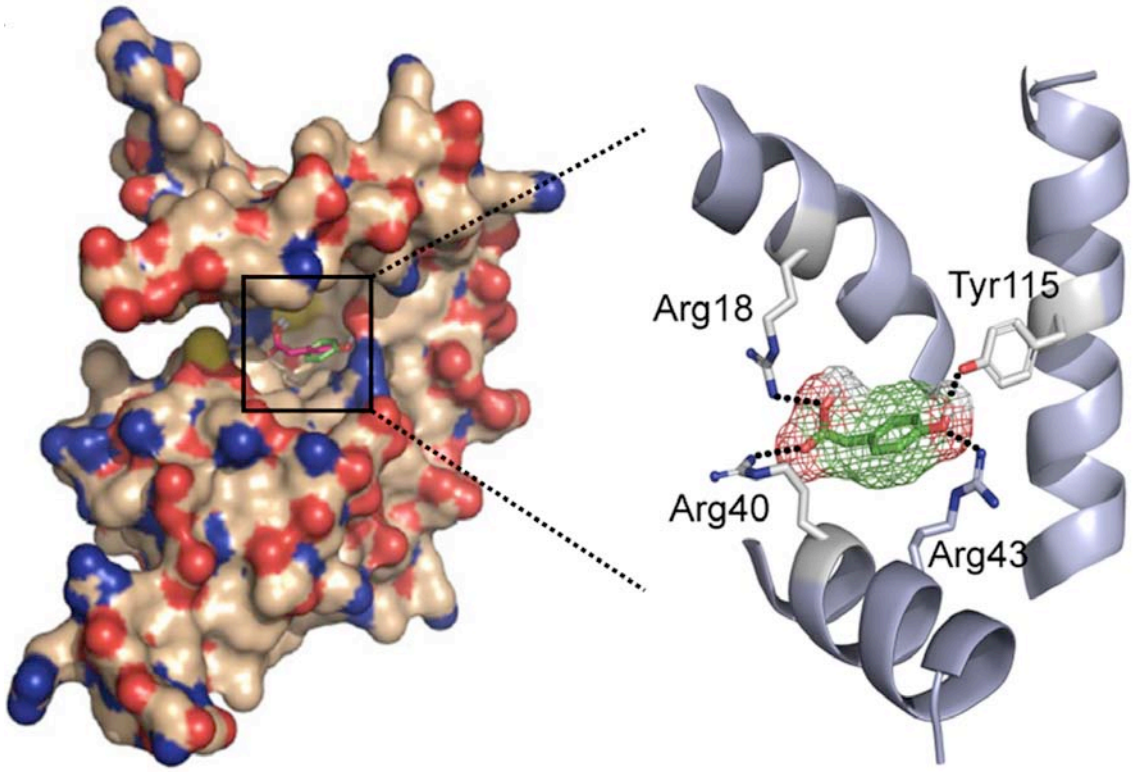


Figure 2.2.3 - Key residues in the 4HPA binding pocket.
Tridimensional surface model of the structure of the NadR protein, obtained as described in [8]. The binding pocket for the 4-HPA molecule is illustrated in the main model and in the magnification showing residues putatively involved in 4HPA binding.

2.2.4 *In vivo* behaviour of selected site directed NadR mutants

Total lysates from MC58 strains expressing different forms of NadR were subjected to Western Blot analyses of NadA expression to assess the ability of each NadR mutant protein to repress *nadA* expression and respond to the 4-HPA inducer. As reported in Figure 2.2.4A, NadR R18A and R40A are unable to repress *nadA* (compare lines 5 & 9 to lines 1 & 3) and mimic the behavior of the Δ1843 strain in the presence and absence of 4-HPA. These results suggest that both substitutions affect the ability of NadR to repress the *nadA* promoter, likely by preventing DNA binding.

The protein NadR W39A presents a lower expression level compared to wild type NadR (compare line 7 to lines 1 & 3) which indicates that the stability of the protein is compromised by the substitution. Possibly as a result, NadR W39A is unable to repress *nadA* expression *in vivo* and exhibits a behavior similar to that of the Δ1843 strain. Interestingly, the addition of 4-HPA increases the expression level of NadR R39A thus suggesting that 4-HPA still interacts and stabilizes NadR W39A (compare lines 7 & 8).

In contrast to R18A, W39A and R40A, the substitution of Tyr_{115} by Ala does not abrogate the *in vivo* repressive function of the protein. Interestingly, NadR Y115A represses more efficiently *nadA* than NadR WT thus suggesting that NadR Y115A acts as a hyper-repressor of NadA expression (compare line 11 to lines 1 & 3). Moreover, the addition of 4-HPA does not affect the repressive activity of NadR Y115A (compare line 11 to lines 2 & 4). Taken together, these data reveal that the Y115A substitution compromises the response of the protein *in vivo* to the 4-HPA inducer without abrogating its ability to repress *nadA*.

In a second round of mutagenesis, we generated a new set of meningococcal strains expressing NadR mutated proteins in the additional residues identified by *in silico* docking (His_7, Ser_9, Asn_{11} and Phe_{25}). We then test their ability to repress *nadA* and sense 4HPA *in vivo* (Figure 2.2.4B). Interestingly, three out of the four new mutations (His_7, Ser_9 and Phe_{25}) lead to a phenotype similar to the one presented by the Tyr_{115}, being able to hyper-repress *nadA* expression (lines 5, 7 and 11 compared to 1) and almost unable to respond to 4HPA (compare lines 5 & 6, 7 & 8, 11 & 12, respectively). The mutant in Asn_{11}, instead, is just slightly affected in its ability to repress *nadA* and is able to sense 4HPA, leading to induction of *nadA* (compare lines 9 & 10 to 1 & 2, respectively).

Altogether, we can conclude that a class of mutation, altering the residues Tyr_{115} , His_7, Ser_9 and Phe_{25}, leads to hyper-repression of *nadA* and greatly affects the 4HPA responsive activity of NadR, suggesting that this group of residues play a role, in the identified binding pocket, in sensing and responding to the ligand.

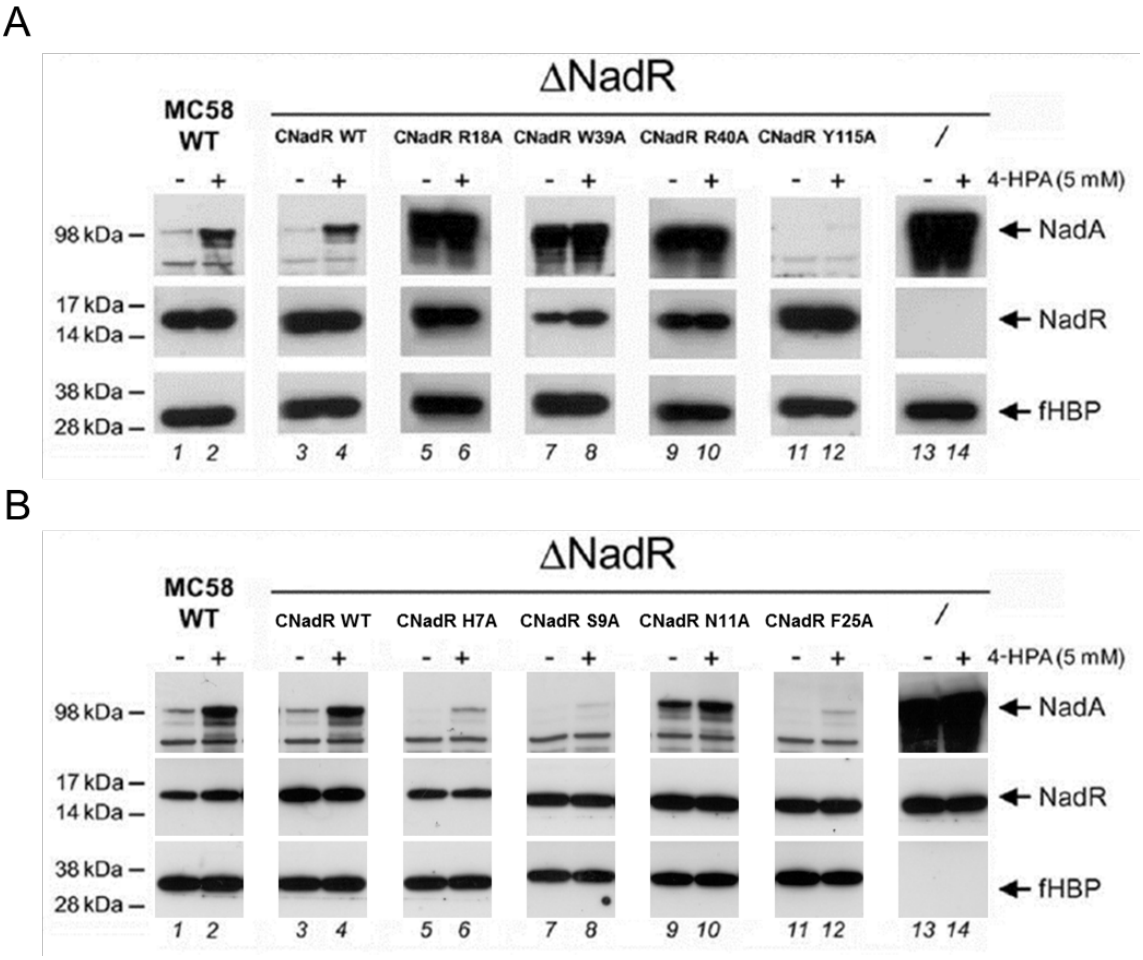


Figure 2.2.4 - *In vivo* characterization of NadR mutants.
(A) Western Blot analysis of the expression level of NadA and NadR in wild type MC58, *nadR* knockout MC58 (ΔNadR) and complemented ΔNadR strains. Cells recovered from an overnight culture on plates were grown in liquid media until mid-log phase (OD600 of 0.5) and then incubated for 1 h in the presence or absence of a fixed concentration of 4-HPA. Cells were harvested and 7 μL of total protein extract from each culture was subjected to SDS-PAGE and Western Blot analysis using anti-NadA, anti-NadR or anti-fHBP antibodies as loading control. (B) The same western blot analyses was performed on a second set of NadR mutants in order to identify more residues involved in 4HPA sensing and responding.

2.2.5 *In vitro* characterization of the DNA- and 4-HPA binding activities of the purified NadR mutant proteins

To further elucidate the behavior of the NadR mutants R18A, W39A, R40A and Y115A, the recombinant proteins were expressed and purified from *E. coli* to assess their *in vitro* DNA-binding activity as well as their response to 4-HPA. Further analyses reported in [8] demonstrated that the solubility, stability and global fold of NadR Y115A and R40A were comparable to that of wild-type NadR, while both R18A and W39A substitutions significantly modify the native structural integrity of the protein, precluding their use in *in vitro* analyses.

To test the binding activity of the NadR proteins *in vitro,* we prepared a radiolabeled DNA probe containing the high affinity binding site OpI from the *nadA* promoter [141]. The binding specificity of NadR WT to the OpI labeled probe was first assessed by EMSA in the presence or absence of increasing amounts of either sonicated salmon sperm DNA (non-specific competitor) or non-labeled OpI DNA (specific competitor). As shown in Figure 2.2.5A (lines 1-3), a slow migrating complex is formed between NadR WT and the OpI labeled probe in the presence of 15 ng protein. The addition of increasing amounts of non-specific competitor does not disrupt the complex (Figure 2.2.5A, lines 4-6), while the use of cold-OpI DNA prevents the binding of NadR WT to the radiolabeled OpI probe in a dose dependent manner (Figure 2.2.5A, lines 7-9). This experiment demonstrates that NadR WT binds specifically to the OpI site of the *nadA* promoter. Similar results were obtained using a labeled probe comprising the high affinity binding site OpII [141] from the *nadA* promoter (data not shown).

We next investigated the *in vitro* ability of NadR Y115A and R40A to bind to the OpI labeled probe. While NadR WT forms a DNA-protein complex with OpI, no interaction could be detected with NadR R40A even at high protein concentrations (Figure 2.2.5B). The DNA-binding activity of NadR R40A is abolished by the substitution indicating that Arg_{40} is essential for DNA binding. Therefore, the *in vivo* defect of this mutant to repress NadA can be directly linked to its incapability to interact with the *nadA* promoter. In contrast to NadR R40A, the Y115A substitution does not abrogate the *in vitro* DNA-binding activity of the protein (Figure 2.2.5B). Interestingly, NadR Y115A binds OpI with a lower affinity compared to NadR WT. The apparent reduction of DNA-binding affinity contrasts with the *in vivo* hyper-repressive activity of NadR Y115A and suggests that the repressive ability of the NadR protein is not exclusively based on its ability to bind the *nadA* promoter DNA. Finally, the addition of 4-HPA does not disrupt the NadR Y115A/DNA complex even at 10 mM concentration (Figure 2.2.5C). *In vitro* experiments to assess NadR binding activity and 4HPA response were performed also with the OpII labeled probe and identical results were obtained (data not shown). Moreover the same results were also confirmed on the whole promoter of *nadA*, comprising multiple binding sites, and using crude extracts instead of the NadR purified protein, to avoid the possibility that unknown factors required for NadR activity were missing in the reaction (Figure 2.2.5D).

Taken together, these data demonstrate that while NadR R40A has lost DNA-binding activity and no longer represses *nadA*, NadR Y115A is a hyper-repressor and is 'blind' to the 4-HPA molecule.

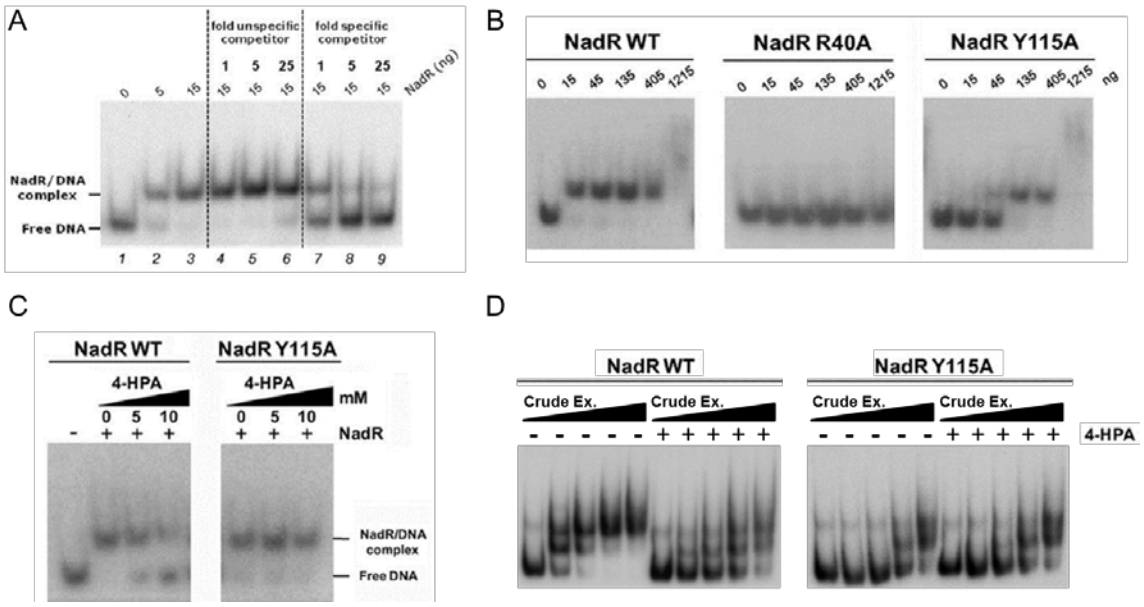


Figure 2.2.5 - Substitution Y115A completely abolishes the *in vitro* effects of 4-HPA.

(A) Gel-mobility shift assays performed with a radioactively labeled DNA probe containing the individual OpI operator in the presence of increasing amounts of purified NadR WT (lines 1-3) and in the presence of either increasing amount of a non-specific competitor (salmon sperm DNA, lines 4-6) or a specific competitor (cold OpI DNA, lines 7-9). The positions of the free and complexed DNA are indicated. (B) Gel-mobility shift assays showing the binding activity of NadR WT, NadR R40A and NadR Y115A on the OpI radio labeled probe. The R40A substitution completely abolishes the DNA-binding activity of NadR while the Y115A substitution reduces the DNA-binding affinity. (C) Gel-mobility shift assay reporting the effects of 4-HPA on the OpI binding activity of NadR WT and NadR Y115A. The presence of increasing concentrations of 4-HPA only induces the release of the DNA probe from NadR WT. (D) Gel-mobility shift assay performed with crude extracts (Crude Ex.) on the whole promoter of *nadA*, comprising multiple binding sites. The effects of substitution Y115A on NadR DNA binding and 4HPA responsive activities are reported (5mM 4HPA is added were indicated).

2.2.6 NadR Y115A does not act as a hyper-repressor on the promoter of *mafA1*

To assess if NadR Y115A also had hyperrepressive activity on other NadR target promoters, namely type II, we further investigated both the *in vivo* behavior and the *in vitro* binding activity of the NadR Y115A on the single operator structured *mafA1* promoter.

Western blot analysis (Figure 2.2.6A) reveals that the MafA1 protein level is not altered *in vivo* by the replacement of NadR wild type with the Y115A. EMSA experiments, performed on the whole promoter of *mafA1* with crude extracts (Figure 2.2.6B), show that NadR Y115A has a reduced binding activity *in vitro* compared to the wild type protein, accordingly with what we saw on the promoter of *nadA*. However, because the binding mode of NadR on type I and type II promoters could be different as demonstrated above (Figure 2.1.9), the reduced *in vitro* binding activity of Y115A results in no detectable binding on promoter of *mafA1 in vitro*, even with high amount of crude extracts. As previously demonstrated (Figure 2.1.7), 4HPA has no effect on NadR binding activity on the promoter of *mafA1*

The fact that the hyper-repressive activity of Y115A is only visible on the type I promoter of *nadA* comprising multiple NadR binding sites, but not on the type II promoter of *mafA1* comprising one single NadR binding site, suggests that this is not an intrinsic property of the mutant protein, rather it is linked to the promoter architecture of the gene which is acting on and could be partly due to an altered tetramerization/multimerization capacity of the mutant.

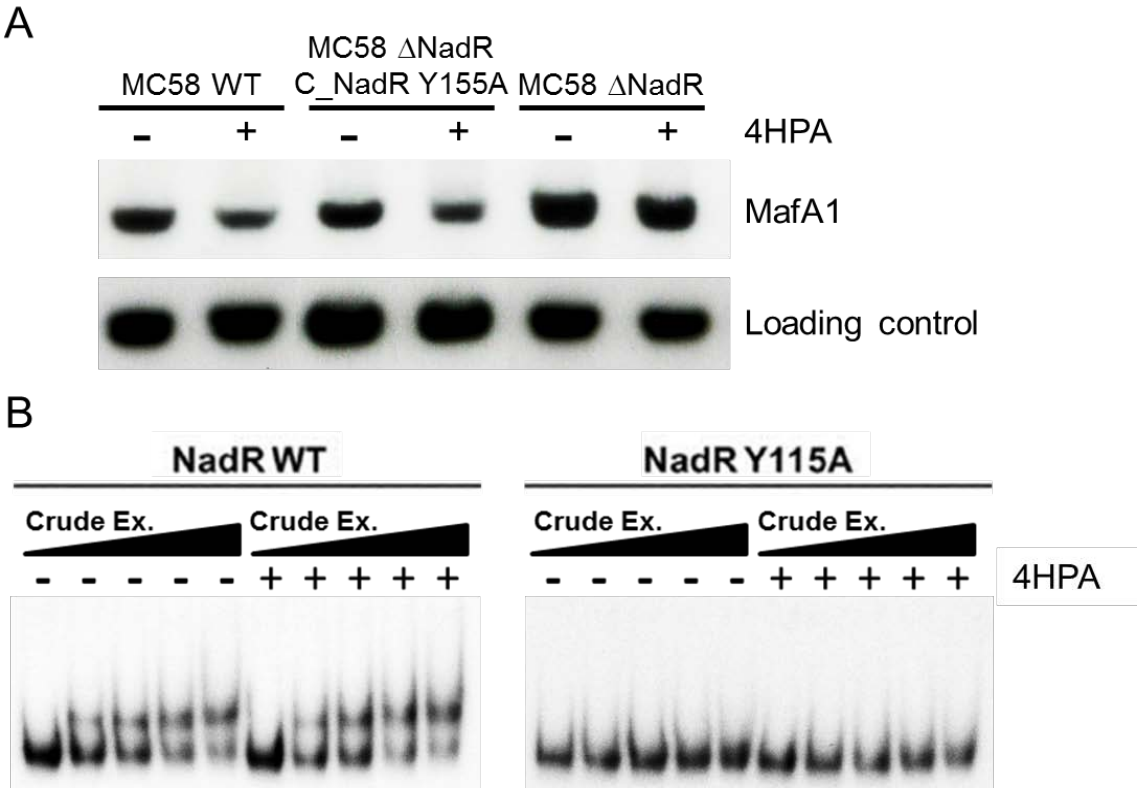


Figure 2.2.6 – *In vivo* and *in vitro* characterization of NadR Y115A on *mafA1.*

(A) Western blot analysis showing the level of expression of MafA1 in presence or absence of either the NadR wild type or the Y115A, as indicated. (B) Gel-mobility shift assay performed with crude extracts (Crude Ex.) on the whole promoter of *mafA1*, comprising only one NadR binding site. The effects of substitution Y115A on NadR DNA binding and 4HPA responsive activities are reported (5mM 4HPA is added were indicated).

2.3 Transcriptional Regulation of the *nadA* Gene Impacts on the Prediction of Coverage of the 4CMenB Vaccine

NadA is one of the major antigens of the 4CMenB vaccine, therefore the regulation of its expression impacts on the vaccine coverage prediction. NadA is repressed by NadR under *in vitro* growth conditions. As seen in previous chapters, different approaches have been followed in order to mimic *nadA* expression during host infection, including the use of physiologically relevant molecules and the *ex vivo* model of saliva, under which NadR-repression is relieved.

The aim of this part of the thesis is to investigate whether the level of expression of *nadA* is different between *in vitro* growth condition and during invasive infection using *in vivo* models. We evaluate further how this difference could implicate on current methods for coverage prediction analyses and whether, when taking this into consideration, we could better predict the contribution of NadA *in vivo* to the effectiveness of the 4CMenB vaccine.

2.3.1 Strains with MATS RP ≤ PBT express NadA in an immunogenic form during invasive disease

Litt and colleagues [205] observed that many protein antigens, including the NadA protein, were recognized by antibodies present in sera of children convalescing after meningococcal disease. Importantly, NadA was significantly more strongly recognized by convalescent patients infected with strains carrying the *nadA* gene, than by uninfected control subjects [205]. We have extended this study by subjecting the 14 *nadA* positive isolates, matched to the sera of the Litt study, to Western blot and MATS analysis in order

to visualize the *in vitro* levels of NadA expression of the infecting strain. In Figure 2.3.1A, Western blots reveal that while the regulator NadR is expressed comparably by all strains tested, the levels of NadA are variable, and some strains (Nm067, Nm100 and Nm188) failed to express the protein at detectable levels under the *in vitro* growth condition used. When analyzed by MATS, the level of NadA expression, calculated as relative potency compared to a reference strain (5/99), correlated well with Western blots results (Figure 2.3.1B and Table 2.3.1). As shown, only 5 strains have RP values above the PBT of NadA which is 0.009 (Nm036, Nm037, Nm081, Nm088 and Nm154), while the remaining strains show RP≤ PBT. However, sera from children infected either by strains that failed to express NadA *in vitro* (MATS RP = 0) or by strains with a NadA RP < PBT, nonetheless are able to recognize at least one form of the NadA recombinant proteins used in the dot blot experiments of Litt and colleagues ([205] and Figure 2.3.1C) more efficiently than sera from subjects infected by *nadA*$^{-}$ meningococci.

These data show that strains with MATS RP values below the PBT are nevertheless able to express the NadA protein in an immunogenic form during invasive disease, driving a robust humoral response. This observation suggests that the levels of expression of NadA under *in vitro* growth conditions may differ from, and be lower than, those reached during infection in the human host.

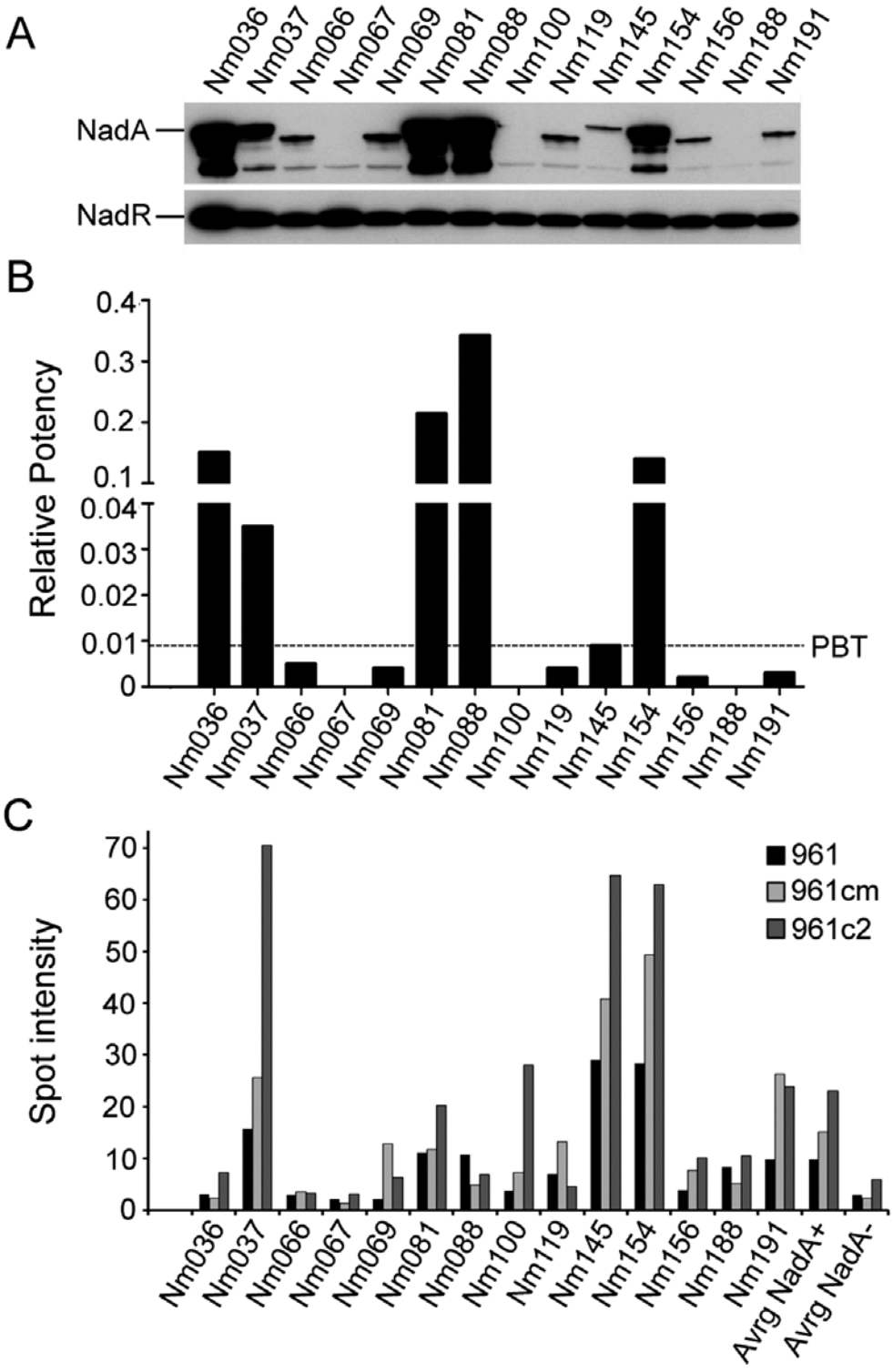

A
Nm036
Nm037
Nm066
Nm067
Nm069
Nm081
Nm088
Nm100
Nm119
Nm145
Nm154
Nm156
Nm188
Nm191
NadA
NadR
B
Relative Potency
0.4
0.3
0.2
0.1
0.04
0.03
0.02
0.01
0
PBT
C
Spot intensity
70
60
50
40
30
20
10
0
961
961cm
961c2
Avrg NadA+
Avrg NadA-

Figure 2.3.1 - Strains with MATS RP ≤ PBT express NadA in an immunogenic form during infection.

(A) Western blot analyses of the wild type *nadA*$^{+}$ strains from the Litt study isolated from convalescing children after meningococcal disease [205], showing NadA and NadR expression. The NadA protein migrates at the molecular weight of about 98 kDa corresponding to the trimeric form of the protein, but varies slightly according to the NadA allele expressed by each strain, while the NadR band migrates at 16 kDa. (B) MATS relative potency (RP) of NadA determined by the MATS ELISA for the panel of isolated strains. A black dashed line represents the positive bactericidal threshold (PBT) for NadA. The RPs of each strain are reported in supplementary table 1. (C) Spot intensity of dot blot experiments adjusted from the study of Litt and colleagues [205]. Reactivities of sera from children infected with the reported isolated strains towards the full length NadA recombinant protein (961) as well as two truncated forms comprising the extracellular portion of NadA (961cm and 961c2) are reported. The average values of reactivity for the 14 *nadA*$^{+}$ strain and the 17 *nadA*$^{-}$ strains from the original study were calculated and reported in the graph as references (Avrg NadA+ and Avrg NadA-, respectively).

Table 2.3.1 - MATS RPs of NadA for *nadA+* strains isolated from patients

	MATS NadA		
Strain	**Low CL**[a]	**RP**[b]	**High CL**[c]
NM036	0.131	0.151	0.174
NM037	0.027	0.035	0.045
NM066	0.003	0.005	0.007
NM067	NA	NA	NA
NM069	0.003	0.004	0.005
NM081	0.173	0.214	0.256
NM088	0.245	0.342	0.501
NM100	NA	NA	NA
NM119	0.003	0.004	0.004
NM145	0.008	0.009	0.013
NM154	0.105	0.14	0.186
NM156	0.002	0.002	0.003
NM188	NA	NA	NA
NM191	0.002	0.003	0.004

a = experimental low confidence limit (StatLIA).
b = relative potency.
c = experimental high confidence limit (StatLIA).
NA = not available.

2.3.2 All strains carrying the *nadA* gene can express high levels of the NadA protein and therefore be killed by vaccine-induced bactericidal antibodies

The main mediator of varying expression levels of NadA within and between strains is the transcriptional regulator NadR, through its ability to differentially repress the phase variant promoter of *nadA*. To understand the relevance of NadR repression to the variable expression levels of NadA observed in different MenB strains under *in vitro* conditions, a representative panel of strains covering a range of NadA expression levels was selected (Table 2.3.2) and the *nadR* gene was deleted in each of them. We evaluated the implications of the alleviation of NadR repression under *in vitro* conditions by Western blot, MATS and SBA analysis (Figure 2.3.2 and Tables 2.3.3 and 2.3.4).

As previously shown [141], all the *nadR*$^-$ strains expressed considerably more NadA than their wild type forebears, confirming that deletion of *nadR* results in strong induction of NadA. Furthermore, all *nadR*$^-$ strains expressed comparable high levels of the NadA antigen measured by Western blot (Fig. 2.3.2A). The MATS assay performed on wild type and *nadR*$^-$ strain pairs demonstrated that the ratio of the RP values for NadA increased from 3 to up to 100-fold in the mutant strains (Fig. 2.3.2B), indicating that all these strains can express high levels of immunogenically relevant NadA antigen when NadR repression is abolished. The MATS assay correlates with the hSBA [243] at values of RP higher than the PBT. Therefore, we compared the ability of immune sera to kill the *nadR*$^-$ knock out strains and their related wild types. Table 2.3.3 shows that sera from mice immunized with NadA alone or with the 4CMenB vaccine have an increased NadA-specific bactericidal activity on *nadR*$^-$ strains compared with wild type strains. The only exception is for strain 5/99, in which, as expected, there is no significant difference in SBA titres

between the wild type and the *nadR*$^-$ strains. In this strain the NadR-mediated repression of NadA is minimal: NadA is highly expressed in the wild type strain. Sera from immunizations with NHBA and fHbp (Table 2.3.3) had invariant activity towards wild-type and *nadR*$^-$ strains, confirming that the knock-out of *nadR* does not alter the susceptibility of these strains in the bactericidal assay and suggesting that neither NHBA nor fHbp expression is regulated in a NadR-dependent way. hSBA assay performed on the mutant strains demonstrates that sera from clinical trial subjects of different age groups immunized with the 4CMenB vaccine formulations were efficiently able to kill all *nadR*$^-$ strains and exhibited extremely high bactericidal titers (Table 2.3.4). Of note, antibodies present in sera from some age groups and apparently ineffective in killing of certain strains (e.g. B3937 and NGP165) show the ability to efficiently kill the equivalent recombinant strains once NadR repression has been relieved.

Taken together these data demonstrate that all the strains carrying *nadA* can potentially express NadA to a level which is sufficient to be recognized and to mediate killing by bactericidal antibody elicited by the 4CMenB vaccine.

Table 2.3.2 - Selected strains used in this analysis

Strain	Clonal complex	ST[a]	Year	Country[b]	Typing	PorA Match OMV_NZ[c]	fHbp ID[d]	NHBA	NadA variant
5/99	ST-8 complex/Cluster A4	1349	1999	N	B:2b:P1.5,2	N	23	20	2
961-5945	ST-8 complex/Cluster A4	153	1996	AUS	B:2b:P1.21,16	N	16	20	2
LNP17094	ST-8 complex/Cluster A4	153	1999	F	B:2b:P1.10	N	16	22	2
B3937	ST-18 complex	6344	1995	D	B:22:P1.16	N	17	23	3
M10574	ST-32 complex/ET5 complex	803	2003	USA	B:NT:P1.7-2,13-1	N	76	3	1
M14933	ST-32 complex/ET5 complex	32	2006	USA	B:ND:P1.22-1,14	N	76	3	1
MC58	ST-32 complex/ET5 complex	74	1985	UK	B:15:P1-7,16b	N	1	3	1
NGP165	ST11 complex/ET-37 complex	11	1974	N	B:NT:P1.2	N	29	29	2

a = multilocus sequence type.
b = AUS, Austria; D, Denmark; F, France; N, Norway; UK, United Kingdom; USA, United States of America.
c = No strains match the PorA P1.4 allele in the OMV_NZ vaccine component.
d = The fHbp allele identification numbers (ID) are reported here according to Oxford database nomenclature.

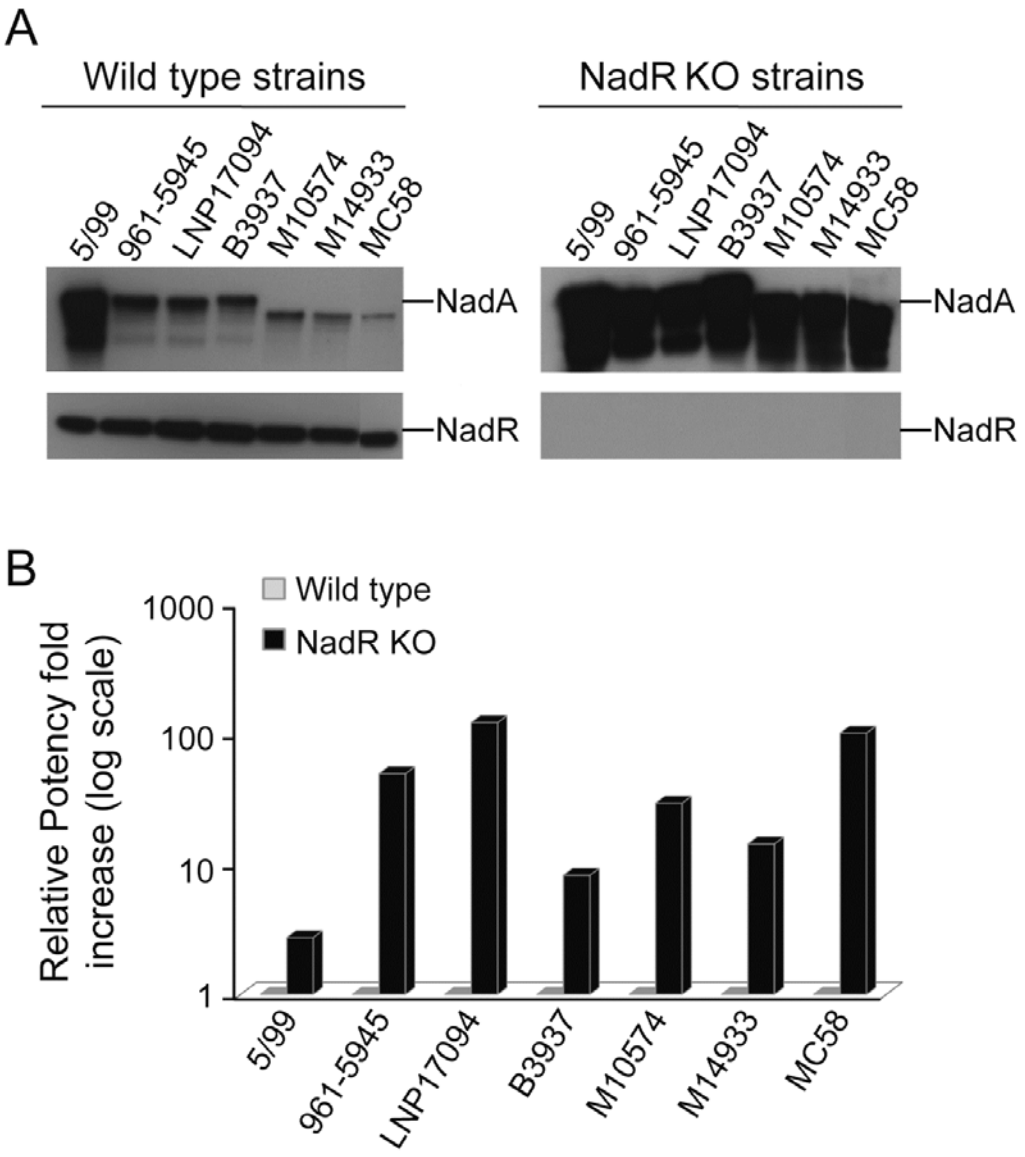


Figure 2.3.2 - NadA espression levels in a panel of wild type and *nadR* knock out strains.

(A) Western blot analyses of wild type and relative *nadR* knocked out strains. The NadA and NadR levels of expression are shown for the representative panel of strains selected and the mutant isogenic strains lacking the *nadR* gene. Different levels of expression of NadA between wild type strains are associated with different phase variants of the *nadA* promoter repressed with different efficiency by the regulator NadR, as previously described [141]. (B) Ratio of MATS RPs of *nadR* knocked out strains versus the wild type strains for the NadA antigen. The RP of wild type strains and the fold increase of NadA RP of *nadR*$^-$ strain, calculated by dividing the RP value of *nadR*$^-$ strains by the RP value of the relative wild type strains, are reported in the graph.

Table 2.3.3 - rSBA performed with immunized mice sera and rabbit complement on wild type and *nadR* knock out strains

Strain	NHBA	fHbp	NadA	4CMenB[a]
5/99	512	<16	>65536	>32768
5/99 *nadR*⁻	128	<16	>65536	>65536
961-5945	1024	1024	1024	4096
961-5945 *nadR*⁻	1024	2048	>65536	>65536
LNP17094	1024	<16	128	4096
LNP17094 *nadR*⁻	512	<16	32768	>65536
B3937	<16	1024	512	2048
B3937 *nadR*⁻	<16	512	>65536	>65536
M10574	4096	128	>8192	>8192
M10574 *nadR*⁻	4096	64	>32768	>32768
M14933	4096	<16	512	>8192
M14933 *nadR*⁻	4096	<16	>32768	>32768
NGP165	128	<16	128	512
NGP165 *nadR*⁻	128	<16	>32768	>32768

a = vaccine formulation with both the three recombinant major antigen and the OMVs, as described in the text.

Grey boxes associate with no killing in the rSBA.

Light green boxes associate with killing in the rSBA.

Dark green boxes associate with significant increased killing in the rSBA, due to NadR knocking out (significant increament = at least 2 dilution titers).

Strains were considered killed if pooled mouse sera achieved an rSBA titer ≥128.

Table 2.3.4 - hSBA on wild type and *nadR* knock out mutants selected strains

Strain	study1		study2	
	Adult		Infant	
	pre-immune	4CMenB post 3[a]	Routine	4CMenB post 4[b]
5/99	<4	256	<4	>512
961-5945	<4	16	<2	16
961-5945 *nadR*⁻	<4	>512	<4	>512
LNP17094	<4	16	<2	16
LNP17094 *nadR*⁻	<4	>512	<4	>512
B3937	<4	8	<2	<2
B3937 *nadR*⁻	<4	>512	8	>512
M10574	<4	32	<2	64
M10574 *nadR*⁻	<4	>512	<4	>512
M14933	<4	16	<2	32
M14933 *nadR*⁻	<4	>512	8	>512
NGP165	<4	<4	2	4
NGP165 *nadR*⁻	<4	>256	4	>256

a = 3 doses of the vaccine given at 0, 2 and 6 months.
b = 3 doses of the vaccine given at 0, 2 and 6 months + 1 boost between 12 and 24 months.
Grey boxes associate with no killing in the hSBA.
Light green boxes associate with killing in the hSBA.
Dark green boxes associate with significant increased killing in the rSBA, due to NadR knocking out (significant increament = at least 2 dilution titers).
Strains were considered killed if pooled sera from different age groups who received the 4CMenB achieved an SBA titer ≥8.

2.3.3 NadA expression can be induced *in vitro* by different physiologically relevant signals

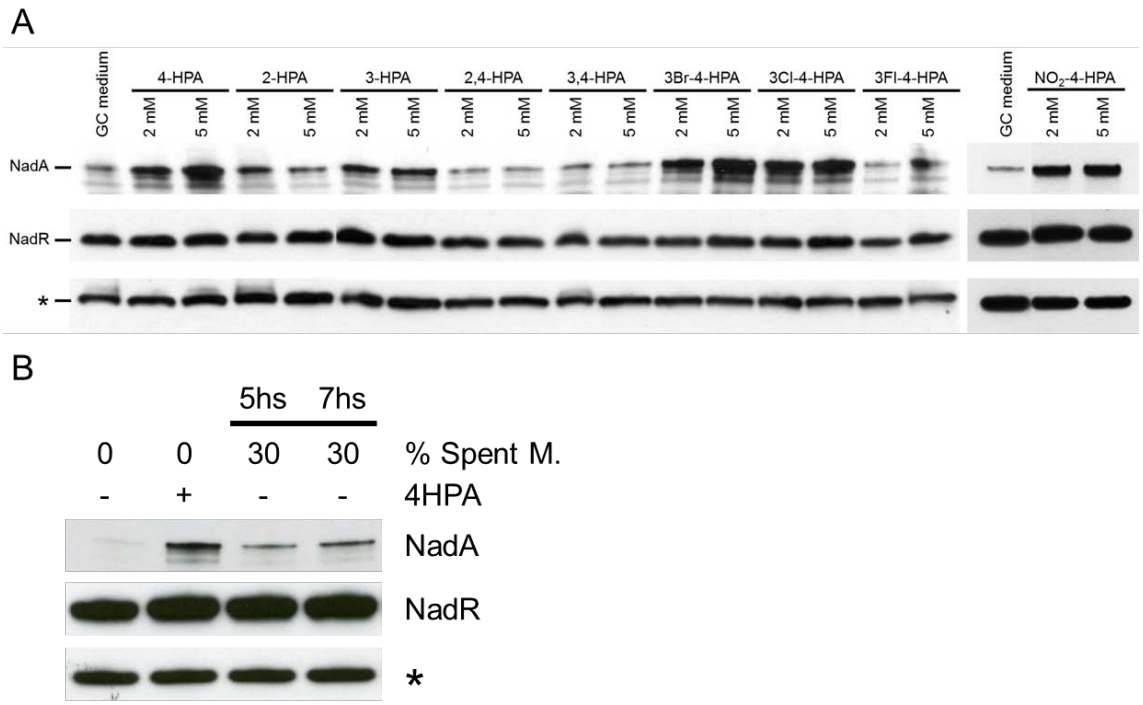
It has been previously shown that NadR-mediated repression of the *nadA* promoter can be alleviated by 4-HPA, a catabolite of aromatic amino acids which is commonly found in human saliva [141, 147]. As seen above, human saliva has been shown to induce NadA expression to the same level as 4-HPA in strain MC58, suggesting that *in vivo* the *nadA* gene might be induced by signals present in saliva [259-261].

We used the substrate binding pocket, involved in the interaction of the 4-HPA ligand and the NadR repressor, represented in Figure 2.2.2, for *in silico* docking experiments to

screen a number of molecules structurally similar to 4-HPA, in order to identify candidates for other potentially physiologically relevant inducers of NadA expression. Any molecule that was identified *in silico* as able to dock in the binding pocket of NadR was tested for its ability to induce NadA expression in MC58 in *in vitro* grown cultures (Figure 2.3.3A). Among the molecular species tested, some (2-HPA, 2,4-HPA and the 3,4-HPA) were unable to induce NadA expression, while others (3Cl4-HPA, NO_2-4-HPA, 3Br4-HPA and, to a lesser extent, 3-HPA) increased the level of expression of NadA to a comparable level to 4-HPA itself. We then verified which of the newly found inducers might have a significant role during meningococcal infection. Interestingly, 3Cl4-HPA, which is structurally similar to 4-HPA (Figure 2.3.4A), has been shown to be produced during inflammatory processes as a catabolite of chlorinated aromatic amino acids [262], while under acid condition or in the presence of polymorphonuclear leukocytes (inflammation), the 4-HPA is nitrated to 4-hydroxy-3-nitrophenylacetic acid (NO_2-4-HPA) [262]. Therefore these molecules represent possible natural ligands that the meningococcus might encounter during infection of the host.

We also assess the hypothesis that meningococcus itself can produce a signal which is able to induce *nadA* expression. In order to do this we recovered the medium of an *in vitro* growth (spent medium) and use it to try to induce *nadA* in MC58 strain. Figure 2.3.3B shows that the spent medium is able to induce the expression of the NadA, even if to a lesser extent than 5mM 4HPA. This could be probably due to the higher dilution of the signal molecule in the spent medium.

Altogether, these results suggest that during infection, multiple signal molecules, either produced by the host (4HPA, 3Cl4-HPA, NO_2-4-HPA or an unknown molecule in saliva) or by meningococci themselves, could lead to the induction of *nadA*.



2.3.3 – Identification of novel *nadA* inducers.

(A) Western blot analyses of the level of expression of NadA in the MC58 strain growth in absence or presence of small molecules. Mid-log cultures of MC58 were incubated for one hour with 2 or 5 mM of the indicated small molecules in order to check *in vivo* the NadA inducing activity of the compounds. (B) Western blot analyses of nadA expression in the MC58 strain following incubation with 30% spent medium recovered after 5 or 7 hours liquid cultures. In both experiments the NadR as well as the fHbp levels of expression are also reported. The fHbp is used as a gel loading control and indicated with an asterisk in the figure. To note that none of the small molecules have an evident effect on either the NadR or the fHbp expression levels.

2.3.4 NadA induction in the selected strain NGP165

The 4CMenB vaccine has been formulated in order to confer protection by targeting multiple antigens on the surface of as many strains as possible. To evaluate the contribution of NadA to vaccine coverage and to test the hypothesis that levels of NadA expression *in vitro* could underestimate the predicted efficacy of bactericidal antibody in mediating the killing of NadA positive strains during infection, we selected strain NGP165 for a case study. NGP165 is mismatched to 4CMenB for fHbp and PorA (carrying fHbp variant 3.29 and PorA serosubtype 1.2, while the vaccine comprises fHbp variant 1.1 and PorA serosubtype 1.4), and with respect to NHBA, it has MATS RP below the PBT and almost negative rSBA titers in the preclinical studies (Table 2.3.3). Thus only the NadA antigen could plausibly contribute to 4CMenB-induced antibody-mediated killing of this strain.

Due to their physiological relevance we decided to test both the 4-HPA and the 3Cl4-HPA molecules in *in vitro* assays using NGP165 selected strain, in order to assess the putative level of NadA expression in the host.

Figure 2.3.4B shows NadA expression is induced by both 4-HPA and 3Cl4-HPA to a similar level, with no statistically significant difference seen between the inductions achieved by the two molecular species in three biological replicates. As previously reported for strain MC58 [259-261], human saliva from different donors is able to induce NadA expression in a dose-dependent manner, to the same extent as 4-HPA (or 3Cl4-HPA, data not shown) in NGP165 (Figure 2.3.4C).

The use of HPA derivatives in *in vitro* assays achieves levels of NadA expression similar to *ex vivo* human saliva and may mimic the predicted levels in the host.

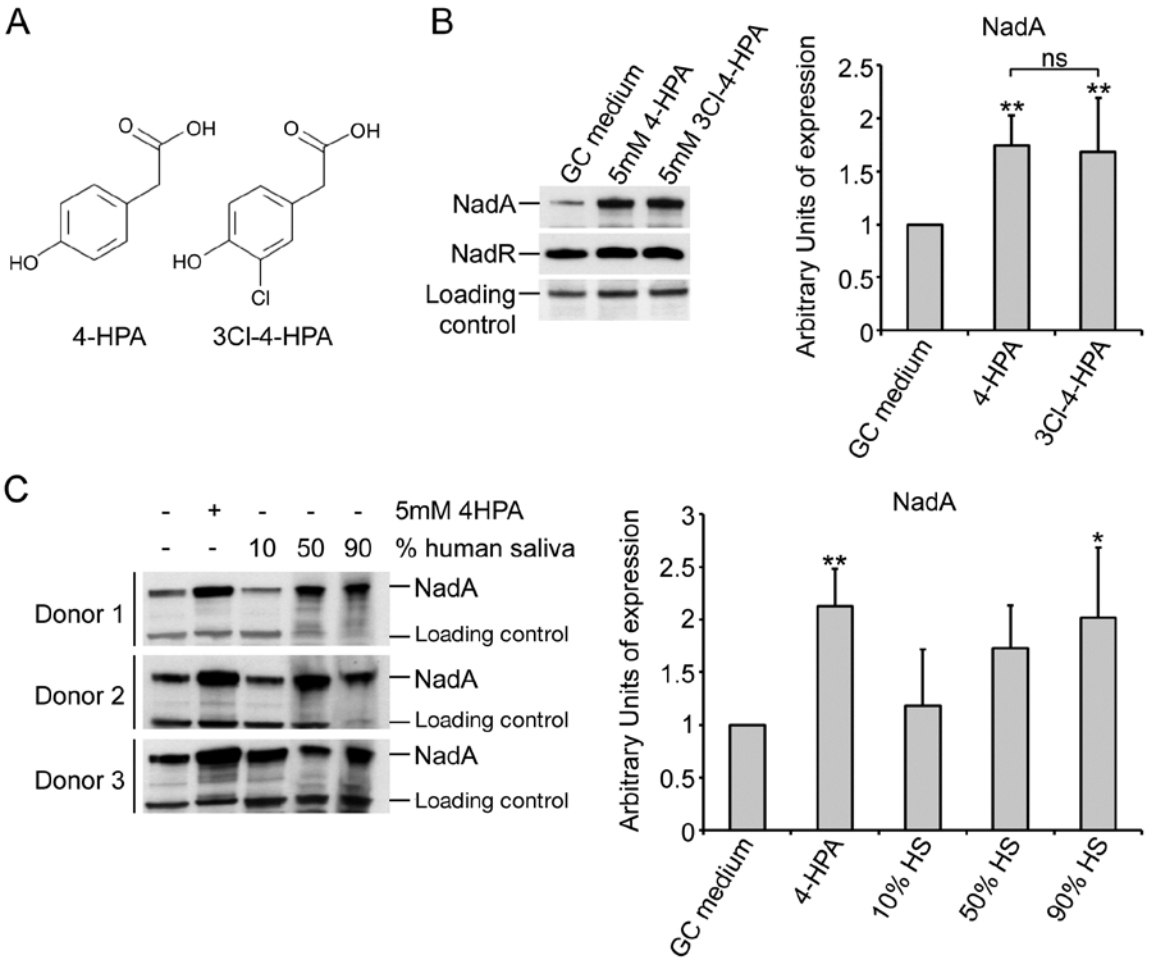


Figure 2.3.4 - Induction of NadA by different physiologically relevant signals in the NGP165 selected strain.

(A) Representation of the chemical structure of the 4-HPA and the 3Cl4-HPA compounds. (B) Western blot analyses of the level of expression of NadA in the NGP165 strain. The wild type NGP165 strain was grown for about two hours to reach mig-log phase of growth and then incubated with either the 4-HPA or the 3Cl4-HPA molecule for one hour to assess the induction of the NadA expression compared to the basal level of the *in vitro* growth in GC alone. The experiment was repeated with three biological replicates and the Western blot bands were quantified using a loading control as reference (fHbp). One representative Western blot is reported together with the histogram summarizing the results of the three replicates. A one-way non parametric ANOVA followed by a Bonferroni post test were performed to evaluate the statistical significance of results. The levels of NadA expression are significantly higher in the presence of the 4-HPA and the 3Cl4-HPA molecules with respect to the basal level in GC (**= pval < 0.01), but not statistically different between them (ns = not significant). (C) Induction of the NadA expression in the NGP165 strain with human saliva (HS). On the left, Western blot analyses of total protein of mid-log cultures of NGP165 incubated for 1 hour with either 4-HPA (lane 2) or with increasing amount of human saliva from 3 different donors (lanes 3, 4 and 5), diluted (V/V) as indicated. The levels of expression of NadA

are shown. On the right, histogram reporting the average values of NadA expression from 5 independent experiments, after quantification of the band signals. Western blot bands were normalized for a non-specific band, indicated as loading control on the right panel, in order to avoid non 4-HPA- or NadR-dependent effects on NadA expression due to possible protein degradation in saliva. A one-way non parametric ANOVA followed by a Bonferroni post test were performed. * = pval < 0.05, ** = pval < 0.01 in the graph, comparing all condition to the basal GC medium level. Comparable level of NadA induction to 4-HPA and saliva were obtained with the 3Cl4-HPA molecule (data not show).

2.3.5 In NGP165, neither 4HPA nor 3Cl-4HPA have any effect on the expression of the other major antigens of the 4CMenB

In order to be sure that the effects that we could see in NGP165 following *nadA* induction with either 4HPA or 3Cl-4HPA would be only due to the increase of the amount of NadA, we assess the effect of these molecules on NHBA and fHbp, the other two major antigens in the 4CMenB vaccine. According to the regulon of NadR that we identified, these two molecules are not regulated by this repressor. However a NadR-independent, HPA-dependent effect on their expression cannot be excluded.

Figure 2.3.5 demonstrates that the HPA molecules do not alter the expression of either NHBA or fHbp.

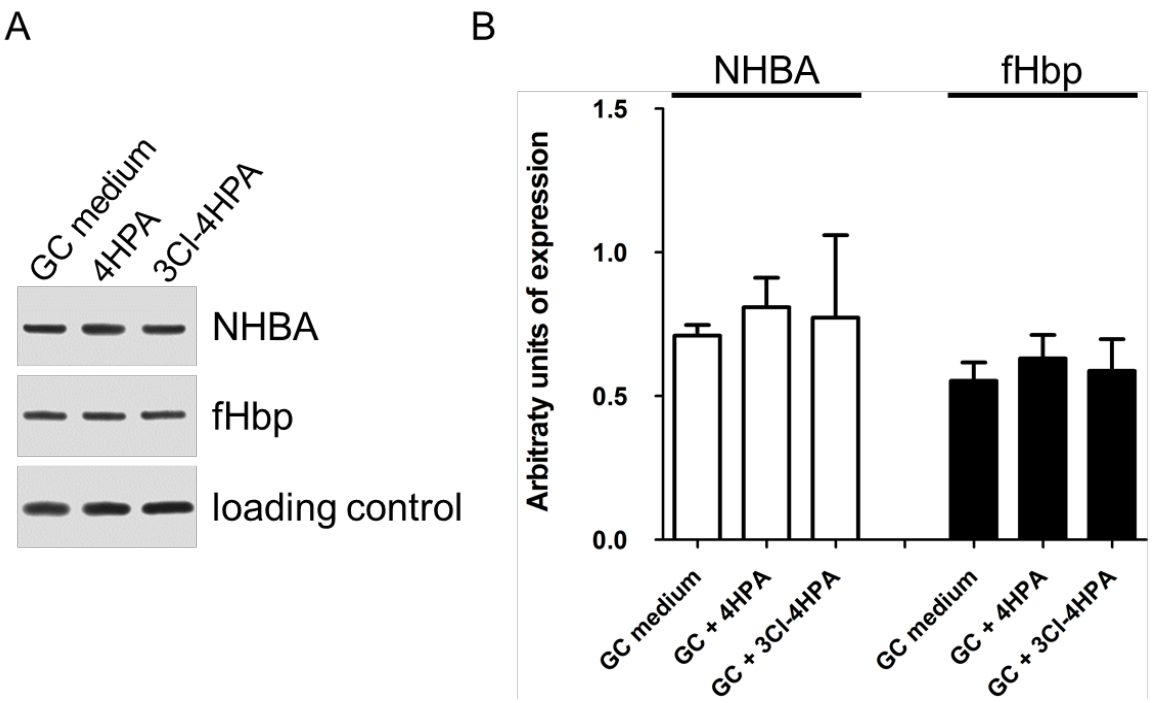


Figure 2.3.5 – Expression of NHBA and fHbp in NGP165 in response to *nadA* inducers

(A) Representative Western blot analyses of the level of expression of NHBA and fHbp in the NGP165 strain, in response to 4HPA and 3Cl-4HPA, as indicated. The constitutively expressed RNA chaperon Hfq has been used as a loading control. The experiment was repeated three times with independent biological replicas. (B) Histogram reporting the average values of NHBA and fHbp expression from 3 independent experiments, after quantification of the band signals. Western blot bands were normalized on Hfq. Standard deviations are reported. A one-way non parametric ANOVA followed by a Bonferroni post test were performed, but no statistically relevant differences were seen neither for NHBA nor fHbp in different conditions.

2.3.6 hSBA and MATS performed with 3Cl4-HPA predict 4CMenB vaccine coverage of the NGP165 strain

We performed the hSBA and the MATS assays on the NGP165 wild type and the *nadR* knock out mutant grown in the absence or presence of 3Cl4-HPA (Table 2.3.5). The NGP165 wild type strain, expressing low levels of NadA *in vitro* (Figure 2.3.4B), has a NadA MATS RP = 0.005, below the PBT for NadA (0.009). Results of the hSBA demonstrated that NGP165 was resistant to killing by pooled sera from infants who received 4 doses of 4CMenB. When grown in the presence of 3Cl4-HPA, the MATS RP of NGP165 increased to

0.028 (Table 2.3.5) and the strain was rendered susceptible in the hSBA using the same infants' sera. Bactericidal titers increased from 4 with pre-immune sera to 128 with immunized sera (Table 2.3.5). As seen for other strains tested, a more pronounced increase in NadA expression was seen in the *nadR*$^-$ mutant, in which the *nadA* gene is fully de-repressed (NadA MATS RP = 0.503). This situation correlates with positive bactericidal titers of >256 in hSBA.

In conclusion, using a modified *in vitro* growth protocol (with HPA supplementation) that we consider more accurately reflects the level of NadA expression that occurs *in vivo*, the MATS and hSBA assays predicted that NGP165 would be efficiently killed during infection by anti-NadA antibodies in sera of subjects immunized with the 4CMenB vaccine.

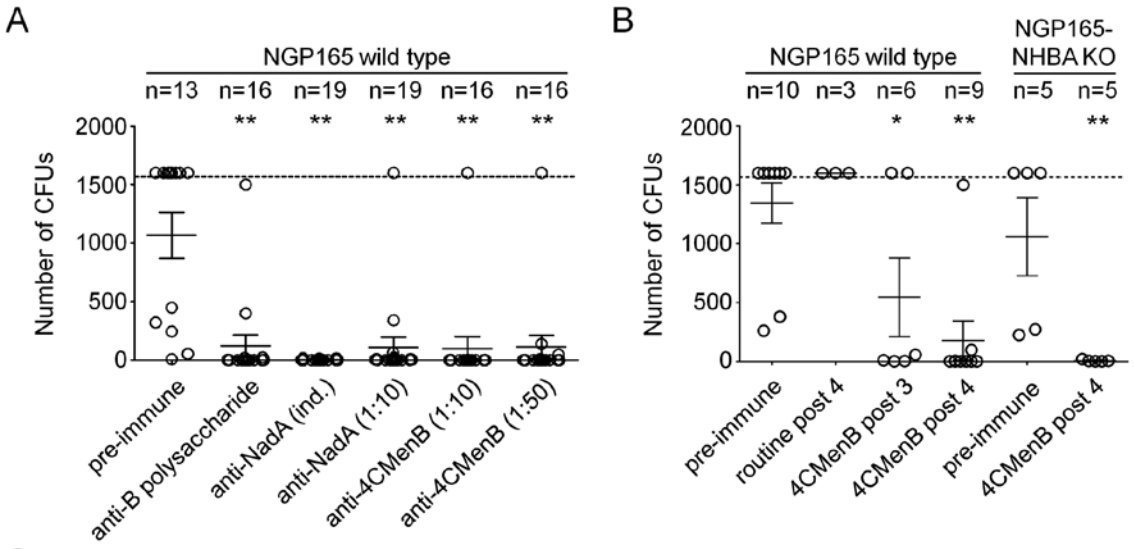
Table 2.3.5 - hSBA and MATS of the NGP165 strain performed with the 3Cl4-HPA molecule

Strain	Inducer	NadA MATS RP	hSBA	
			study3	
			Infant	
			routine	4CMenB post 4
NGP165	none	0.005	2	4
NGP165	3Cl4-HPA	0.028	4	128
NGP165 *nadR*$^-$	none	0.503	2	>256

Grey boxes associate with either MATS RP below the PBT (which is 0.009 for NadA) or no killing in the hSBA. Green boxes associate with either MATS RP above the PBT or killing in the hSBA.
Strains were considered killed if pooled sera from infants who received three immunizations plus one booster of 4CMenB achieved an SBA titer ≥8.

2.3.7 Sera from 4CMenB-immunized infants protect infant rats from infection with strain NGP165.

To determine whether NGP165 would be killed *in vivo* by anti-NadA antibodies, we performed a passive protection assay in the infant rat model [263]. Figure 2.3.6 reports the results of these experiments. Groups of infant rats were inoculated i.p. with an infectious dose (10^5 CFU) of NGP165 after being treated with control serum, or pre- or post-immune sera from mice and infants immunized with either NadA or the 4CMenB. Administration of pre-immune sera from either mice or human infants had no effect on the ability of NGP165 to infect infant rats: 10^5 CFU of NGP165 led to sustained infection in all but one of the 19 animals tested. Sera from mice or human infants immunized with the 4CMenB vaccine formulation conferred protection on the infant rats as well (15 out of 16 and 4 out of 4 rats respectively protected in two experiments). The same human sera results in protection of infant rats from infection with a NGP165 NHBA KO strain (4 out of 5 rats), demonstrating that killing of the strain is not due to anti-NHBA antibodies present in the sera. Taken together these data suggest that in this *in vivo* model NadA is expressed to a sufficient level to be recognized by specific anti-NadA antibodies elicited by NadA in the 4CMenB vaccine and to mediate killing of the bacterium.



C

	Strain	Immunization	Uninfected rats (<10 CFU)
Mouse sera	NGP165 wild type	pre-immune	1/13
		anti-B polysaccharide (2 μg)	12/16
		anti-NadA (ind.)	15/19
		anti-NadA (1:10)	13/19
		anti-4CMenB (1:10)	15/16
		anti-4CMenB (1:50)	12/16
Infant sera	NGP165 wild type	pre-immune	0/10
		routine post 4	0/3
		4CMenB post 3 (1:2)	3/6
		4CMenB post 4 (1:2)	7/9
	NGP165-NHBA KO	pre-immune	0/5
		4CMenB post 4 (1:2)	4/5

Figure 2.3.6 - Passive protection in the *in vivo* rat model.

(A) Plot of the number of CFUs counted for each infant rat, alternatively injected with either mice preimmune sera or mice immunized sera, as indicated below the chart. (B) Plot of the number of CFUs counted for each infant rat, alternatively injected with either pre-immune human sera or sera from human immunized with the 4CMenB vaccine, as indicated. Infant rats were infected with either NGP165 wild type or NGP165 NHBA KO Circles indicate single infant rats, while solid horizontal black lines indicate the average of CFUs counted for each condition, error bars are also reported. A horizontal dashed line indicates the limit of quantification of the CFUs. (* = p val < 0.05, ** = p val < 0.01, comparing all conditions to rats injected with pre-immune sera). No statistical difference is present between the protection of infant rats from infection of either NGP165 wild type or NHBA KO strains. (C) Table showing the results obtained in the in vivo passive protection model.

2.3.8 The promoter of *nadA* is activated *in vivo* during infection of the infant rat model

In order to directly evaluate the expression/induction of *nadA* during infection, we generated reporter strains carrying the promoterless luciferase operon (negative control) or carrying the operon under the control of the *nadA* promoter (P*nadA*-*lux*). The bioluminescence of the resulting strains was evaluated in *in vitro* experiments. Interestingly, the P*nadA*-*lux* strain is significantly less bioluminescent than the negative control (data not shown), indicating that the *nadA* promoter is efficiently repressed under in vitro conditions and as expected 4HPA-specific induction of P*nadA*-*lux* (10-fold) and the derepression of P*nadA*-*lux* in the NadR KO background (385-fold) was observed (Figure 2.3.7A). The infective dose of 10^4 CFU of the negative control and the P*nadA*-*lux* reporter strains were used to infect groups of 5 infant rats and images of ventral views of intraperitoneally infected rats were collected either immediately or 3 and 24 hours after infection. Immediately after infection, both the negative control and the P*nadA* reporter strains are poorly bioluminescent (less than 1.5-fold change from the background) (dark grey and white circles, respectively in Figure 2.3.7B), however three hours post infection, the P*nadA*-*lux* reporter strain is significantly induced (6-fold on average) while the negative control maintains low levels of bioluminescence (Figure 2.3.7B-C). It is worth noting that in P*nad*-*luxA* infected rats a widespread bioluminescence is observed over the entire rat indicative of bioluminescence from bacteria in a systemic infection. 24 hours after infection the bioluminescent signals from both the negative control and the reporter strains are almost indistinguishable from the background (Figure 2.3.7B). These data produced in the infant rat model of septicemia, indicate that the *nadA* promoter is

induced during infection *in vivo*, suggesting that NadA is expressed during invasive disease.

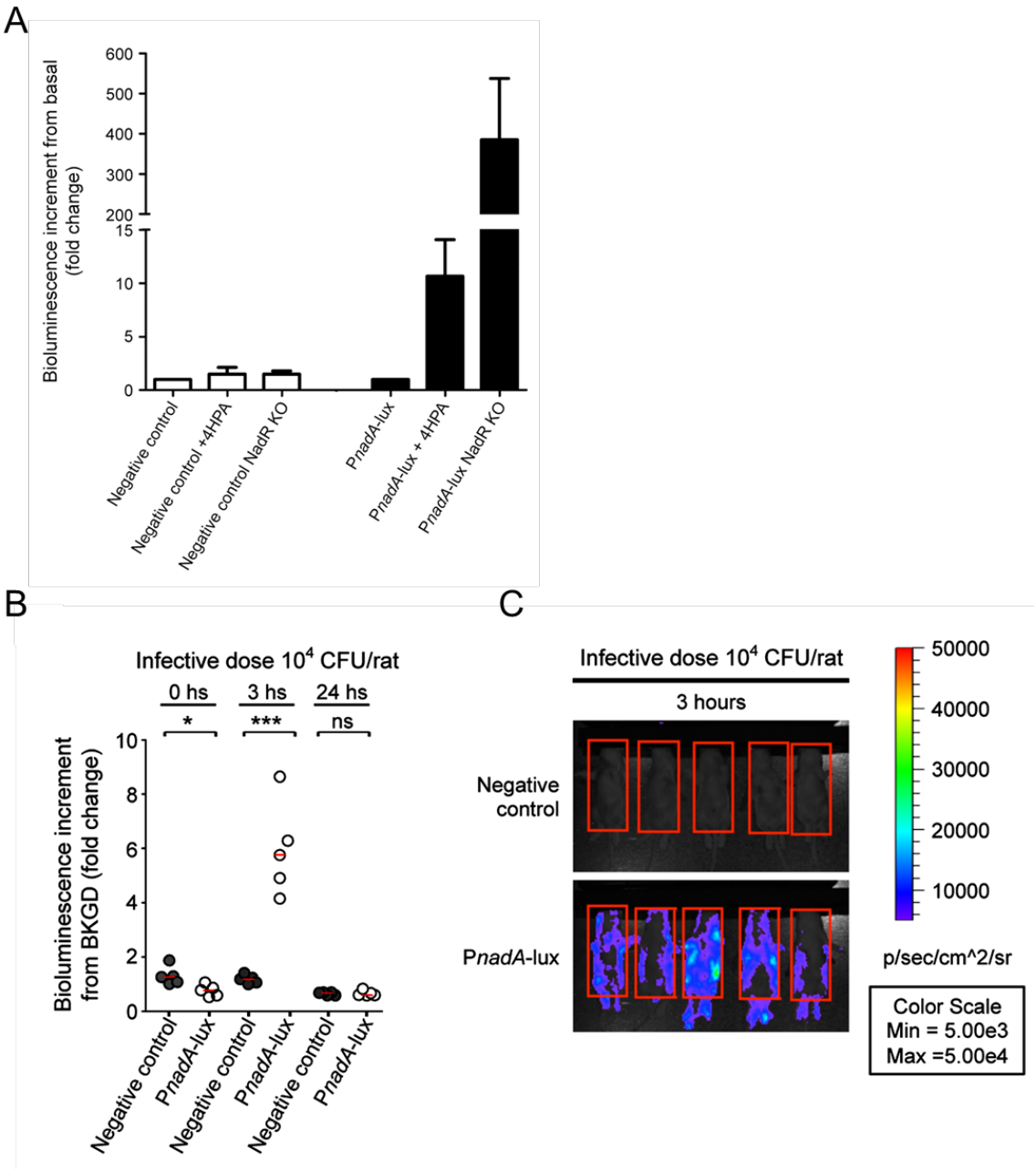


Figure 2.3.7 - Direct visualization of P*nadA* expression in the *in vivo* infant rat model.

(A) Images of serial dilution of liquid cultures of wild type and relative NadR KO strains carrying either the promoterless *lux* operon (negative control) or the P*nadA-lux* reporter, grown in absence or presence of 4HPA, were taken with an IVIS 100 system. Bioluminescence values were obtained after analyzing the images with the Living Image 3.1 software. The basal level of bioluminescence of these strains was obtained by setting to one the bioluminescence of wild type strains observed during growth in GC medium solely. The bioluminescence increment from this basal level was calculated for relative NadR KO strains and during growth in GC in presence of 4HPA and reported in the graph, as indicated (white columns represent the negative control and black columns represent the P*nadA-lux* reporter). (B) Histogram representing the bioluminescence increment from the background (infant rats infected with the 2996 wild type strain) of groups of 5 infant rats infected with 10^4 CFU of the 2996 strain carrying an integrated copy of either the promoterless *luxCDABE* operon (negative control, dark grey circles) or the 2996 *nadA*-lux reporter strain (white circles) as well as median values of bioluminescence increment of each group (solid horizontal red lines) at time points indicated. T-test analyses were performed to assess statistical significant differences between the two groups of infant rats at each time point (* = pval <0.05, *** = pval <0.001, ns = not significant). (C) Panel of ventral views of groups of 5 infant rats infected with 10^4 CFU of the negative control or the P*nadA-lux* reporter, as indicated, taken 3 hours after infection. Red boxes indicate the regions of interest (ROI) that were taken into consideration by the Live Imaging software to quantify bioluminescent values. Blood samples were recovered 24 hours after infection and CFU counts confirm that the bacterial load within infant rats infected with different strains was similar.

3 DISCUSSION

3.1 In the NadR regulon, adhesins and diverse meningococcal functions are regulated in response to signals in human saliva

In this first part of the thesis we dissect the role of NadR, a member of the MarR family of transcriptional regulators, in *N. meningitidis* and define its regulon. We show that NadR co-ordinately regulates many genes that all respond to the small signalling molecule 4HPA, a metabolite of amino acid catabolism that is secreted in human saliva. Of the genes with an altered expression profile, NadR had the greatest effect on the *nadA* gene which exhibited >60 fold derepression in the NadR mutant. All other genes in the NadR regulon display much lower expression rate changes, in accordance with the previously described NadR targets [90].

Interestingly in addition to the NadA adhesin, NadR represses 2 *mafA* (multiple adhesin family A) loci of MC58 (NMB0375 and NMB0652), encoding putative adhesins. We have demonstrated that NadR controls the expression of NadA and the MafA1/A2 adhesins in opposing ways in response to the 4HPA inducer molecule. While 4HPA induces NadA expression, it results in co-repression of the MafA1 and MafA2 adhesins in a panel of meningococcal strains. Interestingly, human saliva has the same differential effect as 4HPA on NadA and MafA expression, suggesting that *in vitro* 4HPA could mimic physiologically relevant signaling molecules *in vivo*. The presence of differential regulatory responses mediated by one unique regulatory protein suggests that the roles of the NadA and MafA adhesins are mutually exclusive. This regulatory response may indicate an important molecular switch enabling adaptation of meningococcus to

changing niches: as a consequence of the external changing niche-specific signals, a dedicated group of adhesins (i.e. NadA or MafA) relevant for the colonization of that niche will be expressed.

Moreover, the NadR-repressed glycolytic enzyme encoded by the *gapA-1* gene was recently shown to play a role in adhesion in meningococcus [264] and its homologues in many other systems are well known to function as a surface exposed adhesin [265-270]. Therefore, Gap-A1 may represent a third adhesin under the control of NadR that will be co-expressed with NadA in response to niche signals.

We have demonstrated that the *nadA* and other 4HPA-induced promoters (type I) comprise multiple NadR binding sites while the *mafA1/A2* promoters (Class II) consist of only one binding site for NadR, centred downstream of the promoter sequence. In addition, 4HPA alters the DNA binding activity of NadR at only type I promoters *in vitro*, without affecting the NadR binding at type II. Therefore, both the architecture of the promoter and the nature of the NadR operators at the promoter may direct the response that NadR will effect to the same signal.

We propose a model (Figure 3.1.1) in which the apo-NadR can bind to and repress *nadA* and other 4HPA-induced genes (type I) 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. This model has been previously proposed for both the gonococcal *farAB* promoter [138] and the meningococcal *nadA* promoter [141] and is supported by the binding at these promoters of the Integration Host Factor (IHF), a histone-like protein which was demonstrated to bend DNA upon binding [271] and could facilitate the interaction between NadR dimers bound at different operators. Following

the binding of 4HPA or other biologically relevant signals (Figure 3.1.1, lower panel), the NadR protein can be stabilized in a conformational state which is not able to efficiently bind to type I operators, thus causing the induction of type I genes.

On the other hand the *mafA1* promoter (type II) could be less efficiently repressed by the apo-NadR. However, the 4HPA bound-NadR can still bind at type II binding sites resulting in a more effective repression of type II genes *in vivo*. The interaction of a small molecule with a MarR regulator is normally associated with disruption of DNA binding, however ligand-binding can enhances the DNA binding activity of MarR homologues [129]. In the case of NadR on type II promoters, the 4HPA molecule does not directly increase the affinity of the protein for its binding site (figure 2.1.7C). Therefore, we suggest that the 4HPA mediated co-repression of type II genes is the result of either a less efficient promoter clearance due to occupancy of 4HPA bound-NadR at the type II operator or different binding kinetics at type II operators between the apo- and the 4HPA bound-NadR forms.

All these considerations suggest that, rather than different signals, it seems to be the different nature of operators and promoters to decide the kind or regulation performed by the NadR protein on its different targets.

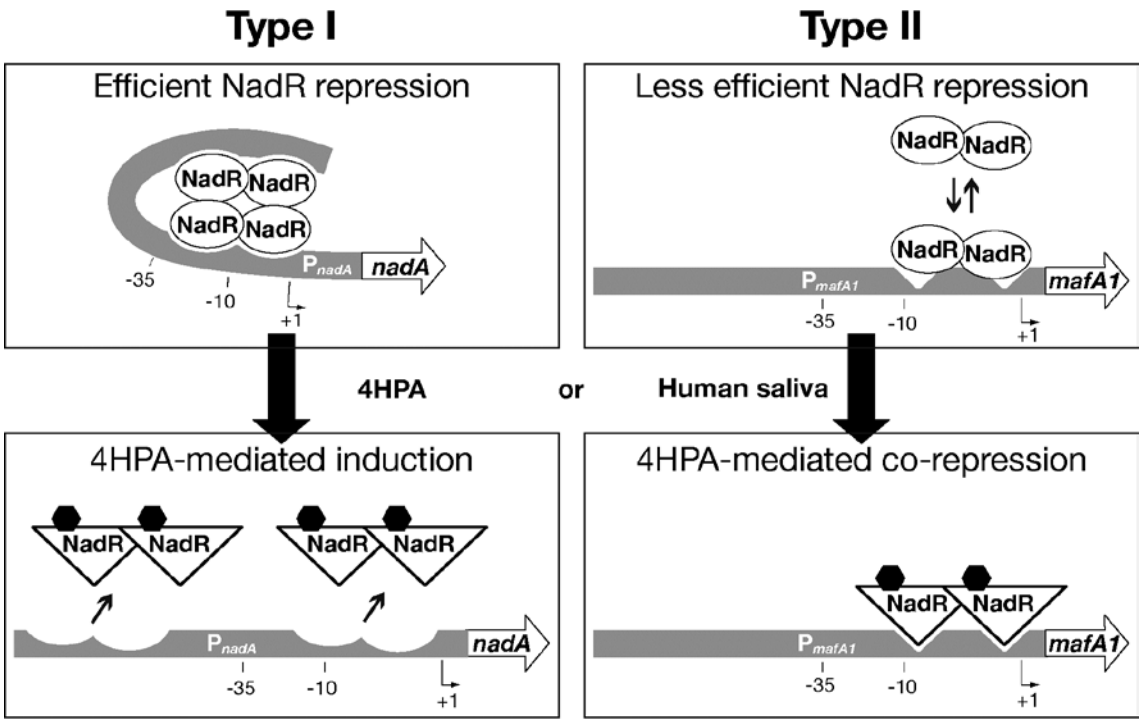


Figure 3.1.1 - Model of NadR regulation on type I and type II gene promoters.

The promoters of *nadA* and *mafA1* are schematically illustrated and represent the type I and type II promoters, respectively. The top panels show the different NadR mediated repression of its different targets. The lower panels show the activity of 4HPA on NadR binding at type I versus type II promoters.

In addition to regulating adhesins, NadR also co-ordinates many other cellular functions in response to 4HPA or similar signals present in saliva, controlling a generalized adaptation of the meningococcus to the host niche colonization. Interestingly, it has been demonstrated that maltodextrins produced in human saliva by α-amylase are crucial for group A streptococcus to successfully infect the oropharynx [272, 273], suggesting that signals present in saliva, other than 4HPA, could be used by bacteria to modulate the gene expression and adapt to the host niche.

NadR is able to repress the expression of various genes implicated in transport of a range of known and unknown substrates, suggesting that it is involved in controlling the substrate-uptake machinery of meningococcus during infection. In addition it also

regulates genes involved in energy metabolic pathways, supporting the idea that it can re-route the metabolism of the cell in response to niche signals. In particular, the ability of NadR of inducing the pathways for amino acid catabolism, such as proline and glutamate utilization, in response to the presence of 4HPA, which is itself an amino acid metabolite, suggests that the environmental availability of amino acid metabolites signals to the cell that amino acids may be abundantly available and NadR adapts the cell metabolism, accordingly. Furthermore, NadR links into other regulatory circuits within the cell by affecting expression levels of transcriptional or post-transcriptional regulators, thus indirectly regulating other targets.

In identifying the NadR regulon and the signals to which it responds we have gained insights into a co-ordinated response of the meningococcus which may be relevant during colonization of the oropharynx. While NadR in meningococcus has evolved away from regulating fatty acid resistance, it coordinates a number of seemingly unrelated functions within the cell in response to a niche-specific small molecule signal. It is tempting to speculate that during colonization of the oropharynx, NadR controls the adhesion properties of the cell, while also adapting metabolism and other physiologically relevant processes to the competitive host niche.

3.2 Structural insight into the mechanism of DNA-binding attenuation of NadR by the small natural ligand 4HPA

After elucidating the global role of NadR, in the second part of the thesis we explored the mechanism by which the 4HPA molecule regulates the NadR DNA-binding activity, on *nadA*, on which, as stated above, NadR had the greatest effect among its target genes. We found that the 4-HPA ligand binds in a pocket located at the junction between the dimerization interface and the DNA-binding lobe. This ligand binding site is mainly formed by structural elements of the two monomers including helix α1 of the first subunit and helices α1 and α5 of the second subunit. Although the number of salicylate sites on MarR molecules is still controversial, superimposition of the NadR/4-HPA complex with available representative MarR/salicylate structures shows that 4-HPA shares a salicylate binding region common to all available models [130-132]. The position of the ligand binding pocket appears therefore well conserved among MarR family members. However the ligand binding site of NadR displays significant variations in amino-acid composition compared to other representative MarR homologues, which suggests differences in ligand specificity. Indeed, NadR does not respond to the broad specific effector salicylate *in vitro* [141] and is unable to associate with the ligand even at high concentrations (our unpublished results). These observations suggest that members of this family have probably evolved separately to respond to distinct signaling molecules thus enabling bacteria to adapt and respond to changing environmental conditions within their natural niches.

The presence of this conserved ligand binding pocket has led to the hypothesis of a shared mechanism of regulation among members of the MarR family. For proteins that

appear to be preconfigured for DNA binding in their apo configuration, the binding of ligands produces a large conformational change that is transmitted to the DNA binding lobes [133]. The distance between the two recognition helices that is essential for association with two consecutive DNA major grooves is altered in the complex thus precluding DNA binding.

The formation of the 4-HPA/NadR complex results in an overall accessibility of the protein similar to the one observed in the absence of the ligand, indicating that 4-HPA likely locks NadR in a conformation incompatible with DNA binding without inducing large conformational changes in the DNA-binding lobes. The binding of 4-HPA induces minor conformational changes in the DNA-binding lobes. This is supported by our mutagenesis experiments, in which the substitution of Tyr_{115} in the ligand binding site alters the dynamics of helix $\alpha 3$ (as reported in [8]) suggesting a cross-talk between elements of the HPA- and the DNA-binding domains. Moreover, the presence of the ligand seems to be not only transmitted to the DNA-binding domain but also to the dimerization interface. Indeed, in the Y115A NadR mutant, 4-HPA accelerates the dimer dissociation rate (data not shown). It is well established that members of the MarR family possess an intrinsic conformational flexibility at the dimerization interface that is exploited on binding of either cognate DNA or ligand to modify the position of the DNA-binding domains [124, 125]. Based on these observations, it is tempting to speculate that 4-HPA makes use of the structural malleability of the dimerization interface to alter the position of the DNA-binding lobes. It seems plausible that 4-HPA reduces the flexibility of the dimerization interface to prevent the motions of the DNA-binding lobes required for interaction with

DNA. On the basis of this model, the binding of the ligand may lock the DNA-binding lobes in place without affecting their global solvent accessibility.

Finally, our mutagenesis data reveal that a class of mutation represented by Tyr_{115} (including His_7, Ser_9 and Phe_{25} mutants) plays a key role in the mechanism of regulation of NadR by 4-HPA. 4HPA can still bind to NadR Y115A, as reported in [8], suggesting that the substitution of this single residue is not sufficient to destroy the ligand binding pocket. However, the Y115A substitution compromises the 4-HPA-mediated stabilization of the complex and prevents DNA dissociation. Taken together, these data suggest that 4-HPA makes use of Tyr_{115} to lock the dimer in a conformation incompatible with DNA binding. Other residues can be involved in the same process and we demonstrated that the substitution of other residues (His_7, Ser_9 and Phe_{25}) leads to a phenotype similar to the one obtained with NadR Y115A, regarding NadA repression and 4HPA response *in vivo*, suggesting that these amino acids can cooperate with the Tyr_{115}.

Taking all these observation into consideration we propose the following model for the 4-HPA-mediated regulation of NadR (Figure 3.2.1). NadR, as other MaR regulators, has an intrinsic conformational flexibility and can pass from a configuration incompatible with DNA binding to a form able to bind DNA, spontaneously and in a reversible way. The binding of 4-HPA in its binding pocket may lock the position of the DNA-binding lobes in place by acting as a “glue” between the dimerization and the DNA-binding domains, thus preventing the cross-talking between the different domains of NadR which is essential to induce the conformational changes required for DNA interaction. The Tyr_{115} plays a key role in this cross-talking mechanism, by being involved in the transduction of the 4HPA binding to the responsive effects in the dimerization domain and therefore in the DNA-

binding lobes. The substitution of the Tyr_{115} causes, indeed, the stacking of NadR onto the DNA and the inability to respond to the 4HPA inducer. The model proposed is based on the comparison of our data with available structural models of regulation of MarR homologs. The resolution of the crystal structure of NadR in its free and ligand-bound state would greatly facilitate our understanding of the mechanism of regulation of NadR by 4-HPA.

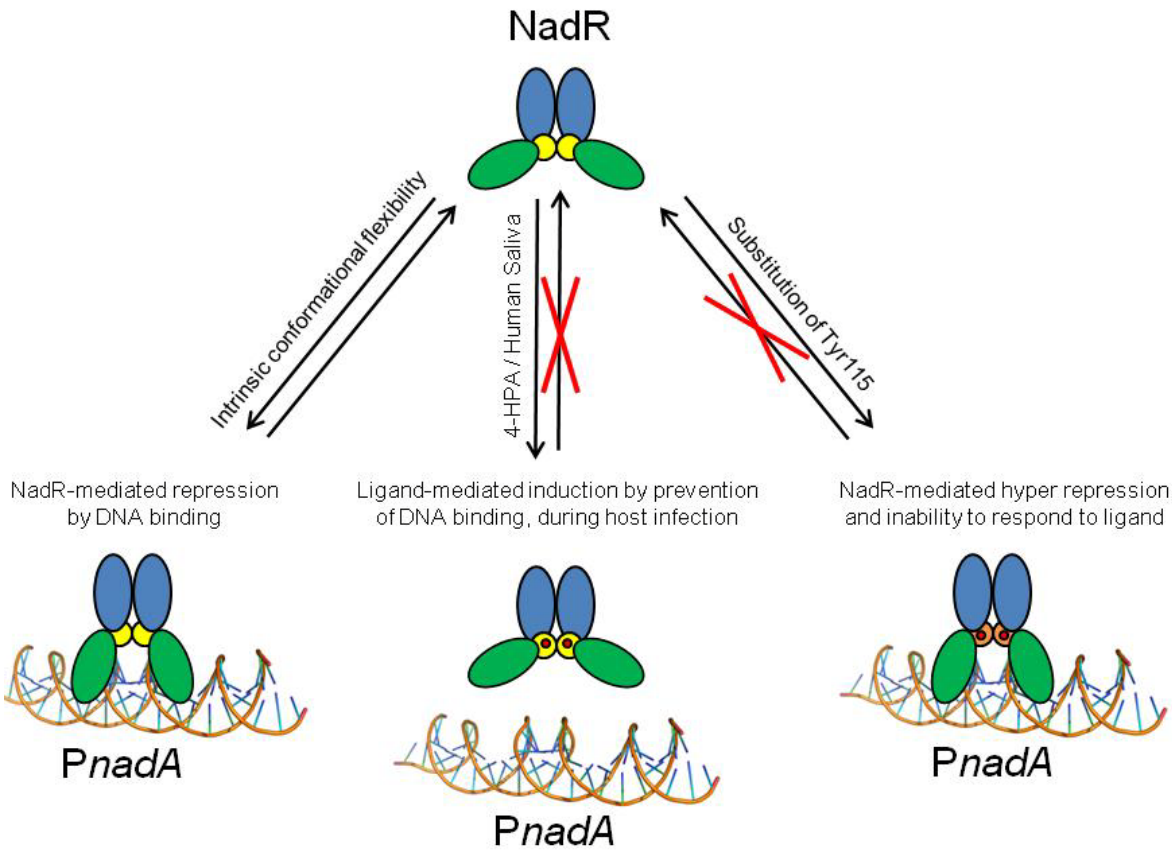


Figure 3.2.1 - Model of NadR DNA binding activity regulation.

The 4-HPA-mediated regulation of NadR on *nadA* is schematized here and fully described in the discussion. In the NadR dimer schematic model, blue ellipses, yellow circles and green ellipses represent the dimerization domain, the ligand binding pocket and the DNA binding lobes, respectively. The altered ligand pocket in the mutated form Y115A of NadR is represented by orange circles. The small molecule ligand is represented by a red dot.

We have demonstrated in the first part of the thesis that NadR regulates a regulon of genes in response to 4-HPA. The MafA1 adhesins are differentially regulated by NadR in response to 4HPA with respect to *nadA*. It is tempting to speculate that, while the conformational modification mediated by 4HPA on NadR is the same as we propose in our model generated on *nadA*, the regulatory outcome may differ due to differential promoter architectures and DNA binding sequences. In fact we demonstrated that, differentially from *nadA*, the Tyr115 mutant has no effect on MafA1 protein level expression, suggesting that the hyper-repressive activity of Y115A on the type I promoter of *nadA* comprising multiple NadR binding sites is influenced by promoter architecture and possibly by an altered tetramerization capacity of the mutant.

3.3 Transcriptional Regulation of the *nadA* Gene Impacts on the Prediction of Coverage of the 4CMenB Vaccine

In the third part of this work, we evaluated the role of NadR regulation on *nadA* expression during infectious disease and therefore the impact on vaccine coverage prediction.

In the absence of an efficacious broadly protective vaccine, MenB is the leading cause of bacterial meningitis and septicemia in many industrialized countries. A novel multicomponent vaccine, 4CMenB, is able to induce bactericidal antibodies against strains expressing vaccine antigens, but because MenB clinical isolates are diverse, it is necessary to evaluate the potential of the vaccine to kill circulating strains and therefore the potential public health impact of this vaccine. The MATS assay, which assesses the relative contributions of the 4 major components present in the 4CMenB vaccine,

predicts whether a given isolate can be killed or not [274]. An evaluation using this assay of more than 1,000 MenB strains from 5 EU countries predicted that 73% to 87% would be covered by 4CMenB. However, the relative contribution of NadA to this combined coverage prediction appers to be low (less than 2%) [246].

The role of NadA in eliciting bactericidal antibodies protecting against circulating strains, has been unclear due to NadR-mediated repression of NadA expression under the *in vitro* growth conditions used for MATS and hSBA assays, which are performed in the early phase of growth, when NadR represses maximally *nadA* expression [141]. For this reason it is reasonable to anticipate that *nadA* expression *in vitro* could be different than the level reached in the host. Litt and colleagues [205] previously showed the presence of antibodies that recognized recombinant NadA in children convalescing after meningococcal disease. Interestingly, we report here that sera from children infected by isolates that failed to express NadA in culture (MATS RP = 0), or express low NadA levels (MATs RP value < PBT) are able to recognize NadA significantly more than sera from subjects infected by *nadA*$^{-}$ strains (Figure 2.3.1). Taken together, these observations suggest that despite the low levels of NadA expression *in vitro*, all these strains express NadA in an immunogenic form in the setting of invasive disease. Furthermore, anti-NadA antibodies are also found in healthy individuals with levels tending to increase with age [275], suggesting that NadA is expressed *in vivo,* at a level sufficient to drive the immune response. NadA expression is also induced in the *ex vivo* model of human saliva (Figures 2.1.6 and 2.3.4), suggesting again high expression in the niche of meningococcal colonization, which can be mimicked *in vitro* by addition of 4-HPA or 3Cl4-HPA representing natural inducers of NadA present in the host.

Using the recombinant *nadR*$^-$ strains and the HPA inducers as a model for *in vivo* expression, we have validated NadA as a potent immunogen in all ages and a valid target for protective responses. Once repression mediated by NadR is relieved and NadA is expressed at high levels, strains normally resistant to killing by 4CMenB immune sera are rendered highly susceptible to killing in SBA. The molecular mechanism of both NadR repression of *nadA* and induction mediated by HPA compounds is conserved in a wide panel of *N. meningitidis* strains belonging to different clonal complexes (Figures 2.1.5 and 2.3.2). These observations suggest that any strain carrying *nadA* could potentially be targeted by bactericidal antibody elicited by the 4CMenB vaccine when NadR repression is relieved during infection.

The ability of passively administered vaccinees' sera to protect mice from infection of NGP165, and to return protective results in the modified MATS and hSBA assays, when NGP165 is grown *in vitro* with added HPA, suggests that this treatment mimics the *in vivo* status of NGP165 with respect to NadA expression. It is noteworthy that passively administered sera from mice immunized with NadA recombinant protein alone protect infant rats from infection with NGP165, demonstrating that the level of expression reached by NadA alone *in vivo* is sufficient to promote bacterial killing. Finally, a bioluminescent *nadA-lux* fusion demonstrates the induction of the *nadA* promoter three hours after infection in the infant rat model, demonstrating the expression of *nadA* during bacterimia *in vivo.*

The expression of NadA *in vivo* in the host is in line with its putative role during meningococcal infection. Its adhesive role is important in the course of colonization of the human upper respiratory tract. Following the passage across the epithelium,

meningococcus invades tissues and blood, where NadA can interact with human blood leukocytes and lead to enhanced immune stimulation [199, 201, 276, 277]. For these reasons, NadA is to be considered an important meningococcal virulence factor, involved in progressively invasive steps of infection. In accordance with this statement we demonstrated here that different signals are able to modulate the NadR repressive activity on NadA (Figure 2.3.3). Signals present in saliva, which we can mimic by using HPA molecules at high concentration during *in vitro* growth, may mediate NadA induction during the colonization of the oro-pharynx. However, because NadA could be required during other steps of the pathogenesis, multiple signals in niches other than the pharynx could modulate NadA expression as soon as the bacterium passes the epithelial barrier. We demonstrate indeed in the infant rat model of bacteremia that NadA is expressed widely three hours post infection and this up-regulation could be due to molecules such as the 3Cl-4HPA and NO_2-4HPA, which are produced by leukocytes during inflammatory processes [262, 278]. Furthermore, we cannot exclude that other molecules can act on NadR by increasing its repressive action on its targets.

From our data, current methods used to predict coverage of strains by the 4CMenB vaccine are underestimating the contribution of the NadA antigen, suggesting that all *nadA*$^+$ meningococcal strains may be susceptible to bactericidal anti-NadA antibodies elicited by vaccination. A more accurate prediction may be obtained by addition of physiologically relevant inducers to *in vitro* grown bacteria, resulting in similar NadA expression levels to those measured in *ex vivo* and *in vivo* models of infection. This study provides new insights into the expression of the NadA antigen during infection, which are

fundamental for the implementation of prophylactic strategies such as vaccines and evaluation of their impact on public health.

4 MATERIALS AND METHODS

4.1 Bacterial strains and culture conditions

The *N. meningitidis* strains used in this study include the wild type MC58, 5/99, 961-5945, LNP17094, B3937, M10574, M14933, NGP165 and 2996 strains, derivatives of these wild type strains and the previously reported clinical isolates NM036, NM037, NM066, NM067, NM069, NM081, NM088, NM100, NM119, NM145, NM154, NM156, NM188 and NM191 [205]. All strains used are reported in Appendix Table 1.

N. meningitidis strains were routinely cultured, stocked and transformed as previously described [279]. They were grown on GC (Difco) agar medium plates supplemented with Kellogg's supplement I at 37°C/5% CO2 overnight (O/N). For liquid cultures, after O/N grown on solid medium, few colonies were inoculated in 7 ml GC broth supplemented with Kellogg's supplement I to an initial optical density at 600 nm (OD_{600}) of 0.05.

When required erythromycin and/or chloramphenicol antibiotics were added to culture media at the final concentration of 5 μg/ml and 20 μg/ml, respectively, isopropyl β-D-1-thiogalactopyranoside (IPTG) were added to achieve final concentrations of 1 mM. 2-, 3-, or 4-Hydroxyphenylacetic acid (2,3, or 4-HPA), 3-Chloro-, 3-Fluoro- or 3-Bromo-4-Hydroxyphenylacetic acid (3Cl4-HPA, 3Fl4-HPA, or 3Br4-HPA), 2,4- or 3,4-Dihydroxyphenylacetic acid (2,4-HPA, or 3,4-HPA) in aqueous solution was added to culture media to achieve final concentrations of 1, 2 or 5 mM. All these compounds were obtained from Sigma-Aldrich with exception of the 3Br4-HPA, which was purchased from Chemsigma.

E. coli DH5-α [280] and BL21(DE3) [281] cultures were grown in Luria–Bertani (LB) medium, and when required, ampicillin, chloramphenicol and/or IIPTG were added to achieve final concentrations of 100, 20 μg/ml and 1 mM, respectively.

4.2 Construction of mutant and complementing strains

DNA manipulations were carried out routinely as described for standard laboratory methods [282]. Total lysates from single colonies of all transformants were used as template for diagnostic PCR analysis to confirm the correct insertion by a double homologous recombination event. The *nadA* promoter was amplified and sequenced in each transformant to ensure that the same numbers of repeats were present as in the derivative strain. The NGP165 NHBA null mutant was generated as described previously [182].

4.2.1 Generation of NadR null mutant and NadR complementing strains

The NadR mutants of the MC58, 5/99 and 961-5945 strains were previously reported [141] and indicated as MC-Δ1843, 5/99-Δ1843, 961-Δ1843, respectively. The NadR mutants of the LNP17094, B3937, M10574 and M14933 strains were generated by transformation of the wildtype strains with pΔ1843ko::Cm [141] and selection on chloramphenicol, leading to the generation of LNP-Δ1843, B39-Δ1843, M10-Δ1843 and M14-Δ1843 strains, respectively.

For complementation of the MC-Δ1843 NadR null mutant [141], the *nadR* gene under the control of the P_{tac} promoter was reinserted into the chromosome of MC-Δ1843 strain between the converging open reading frames (ORFs) NMB1428 and NMB1429, by transformation with pComEryPind-*nadR*. pCompEryPind-*nadR* is a derivative plasmid of

the pSLComCmr [118], in which the *nadR* gene was amplified from the MC58 genome with the primer pair 1843-F and 1843-R2 and cloned as a 441-bp NdeI/NsiI fragment downstream of the P_{tac} promoter. The chloramphenicol resistance cassette of pSLComCmr was substituted with an erythromycin resistance cassette, amplified with primers EryXbaF and EryBamR and cloned into the XbaI-BamHI sites (Appendix Table 3), generating pComEryPind-*nadR*. This plasmid was transformed into MC-Δ1843 for the generation of the ΔNadR_C complemented mutant strain and transformants were selected on erythromycin. The *nadR* gene in the complemented strains was expressed in an Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible manner. A final concentration of 1 mM IPTG resulted in NadR expression levels similar to that of MC58 wild type strain.

4.2.2 Generation of MC58 strains expressing NadR mutated proteins

In order to generate MC58 mutant strains expressing only amino-acid substituted forms of NadR, plasmids containing the sequence of *nadR* alternatively mutated in the codons coding for His_7, Ser_9, Asn_{11}, Arg_{18}, Phe_{25}, Trp_{39}, Arg_{40} and Tyr_{115} were constructed using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). Briefly, the *nadR* gene was mutated in the pComEry-1843 plasmid using 8 couples of mutagenic primers (H7A-F/H7A-R, S9A-F/S9A-R, N11A-F/N11A-R, R18A-F/R18A-R, F25A-F/F25A-R W39A-F/W39A-R, R40A-F/R40A-R and Y115A-F/Y115A-R (Appendix Table 3)). The resulting plasmids were named pComEry-1843H7A, -1843S9A, -1843N11A, -1843R18A, -1843F25A, -1843W39A, -1843R40A and -1843Y115A, and contain a site-directed mutant allele of the *nadR* gene, in which the selected codons were respectively substituted by a GCG alanine codon, and were used for transformation of the MC-Δ1843 strain. The resulting transformed strains

were named MC-Δ1843_H7A, _S9A, _N11A, _CR18A, _F25A, _CW39A, _CR40A and _CY115A, respectively. The *nadR* wild type or mutated genes in the complemented strains were expressed in an Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible manner. A final concentration of 1 mM IPTG resulted in NadR expression levels similar to that of MC58 wild type strain.

4.2.3 Generation of *lux* reporter strains

To generate bacterial luciferase transcriptional fusions of the promoters under study at a chromosomal location between the two converging ORFs NMB1074 and NMB1075, flanked on both sides with transcriptional terminators, plasmid pSL-LuxFla for allelic exchange in *N. meningitidis* strains was constructed. Briefly, the promoterless *luxCDABE* operon and *cat* cassette were subcloned from pSB1075 [283] into pBleuskript II [9] as an EcoRI-BamHI fragment and then cloned as a 6.5 kb XhoI-BamHI fragment into pSL-*furlacZ* [94] replacing 4.7kb containing an erytromycin cassette, and *fur-lacZ* fusion, generating pSL-*lux*-Fla. The *nadA* promoter was amplified with primers NadluxF2/NadluxR2 and cloned as a 250 bp XhoI-KpnI fragment upstream of the *luxCDABE* operon generating pSL-P*nadA*-*lux*. The pSL-*lux*-Fla and pSL-P*nadA*-*lux* plasmids were used for transformation of the MC58, and MC58-Δ1843 strains generating MC58-*lux*, MC58-P*nadA*-*lux*, and MC58-Δ1843-*lux* and MC58-Δ1843-P*nadA*-*lux*, respectively, for the *in vitro* reporter analyses, and the 2996 strain generating 2996-*lux* and 2996-P*nadA*-*lux*, respectively, for the *in vivo* reporter analysis.

4.3 Western blot analysis

N. meningitidis colonies from freshly grown overnight plate cultures were resuspended in GC medium to an optical density at 600 nm (OD_{600}) of 0.05 and grown at 37°C to logarithmic phase (OD600 of 0.5, ca. 2 hours incubation). Samples of 1 ml were harvested and resuspended in 1x SDS-PAGE loading buffer (50 mM Tris Cl pH 6.8, 2.5 % SDS, 0.1 % Bromophenol Blue, 10% glycerol, 5% beta-mercaptoethanol, 50 mM DTT), normalizing the concentration to a relative OD_{600} of 5.

To prepare protein extracts from bacteria incubated with either different small molecules or human saliva, *N. meningitidis* colonies from overnight GC plate cultures were resuspended in GC to an OD_{600} of 0.05. Liquid cultures were grown until mid log phase (OD_{600} = 0.4), harvested and resuspended in either GC, GC + 2mM or GC + 5mM of the indicated molecule or 10%, 50%, 90% (V/V) human saliva in GC containing EDTA-free Protease Inhibitors Cocktail (Roche). Bacteria were then incubated for 1 hour with agitation at 37°C. 1 ml of each liquid culture was harvested and resuspended in 100 ml of SDS-PAGE loading buffer. When required the growth was followed by 1 hour induction at 37°C with 1 mM IPTG.

For Western blot analysis, 10 μg of each total protein sample in 1x SDS-PAGE loading buffer was separated by SDS-PAGE, and transferred onto nitrocellulose membrane using an iBlot Dry Blotting System (Invitrogen). Membranes were blocked overnight at 4° C by agitation in blocking solution (10% skimmed milk, 0.05% Tween-20, in PBS) and incubated for 90 min at 37°C with primary antibodies (anti-NadA [203], anti-NadR [141], anti-Maf [254], anti-fHbp [234] and anti-Hfq [284] polyclonal sera) in 3% skim milk blocking solution. After washing, the membranes were incubated in a 1:2000 dilution of

peroxidase-conjugated anti-rabbit immunoglobulin (Biorad) or anti-mouse immunoglobulin (Dako) in 3% skim milk blocking solution for 1 hour at room temperature and the resulting signal was detected using either the Supersignal West Pico chemiluminescent substrate (Pierce) or the Western Lightning-ECL chemiluminescent substrate (Perkin-Elmer). Quantification of the signals from western blots bands was performed by using a PhosphorImager and ImageQuant software (Molecular Dynamics).

4.4 Protein expression and purification

The expression and purification of the NadR recombinant protein were carried out as fully described previously [141]. For expression and purification of NadR mutated proteins, the sequence of *nadR* was mutated in the expression plasmid pET15-1843 [141] as described above using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) and the mutagenic primers pairs R18A-F/R18A-R, W39A-F/W39A-R, R40A-F/R40A-R and Y115A-F/Y115A-R (Appendix Table 3). The resulting pET15-1843R18A, -1843W39A, -1843R40A and -1843Y115A vectors were subsequently transformed into *E. coli* BL21(DE3) strain for protein expression and purification. Fractions containing the purified proteins were analyzed by SDS-PAGE, pooled and dialyzed overnight against 50 mM Tris-HCl pH 8.0, 300 mM NaCl using a 10-kDa molecular weight cutoff dialysis membrane (Slide-A-lyzer dialysis cassettes, Pierce). The 6xHisTag was cleaved at room temperature using thrombin agarose resin (Sigma Aldrich) and removed with the Ni-NTA matrix. The purity and the identity of each protein were verified by SDS-PAGE and mass spectrometry and the concentrations were measured using the Bradford assay. Purified proteins were aliquoted, flash frozen in ethanol/dry ice and stored at -80°C until use.

4.5 Electromobility shift assays (EMSA).

For gel shift experiments, a probe corresponding to the respective promoter region (150-280 bp) of the first gene in probable operons containing NadR target genes was amplified using primers listed in Appendix Table 3 and named with P (for promoter) and a number according to the NMB annotation. Two probes spanning from -170 to -116 (OpI) and -9 to +81 (OpII) with respect to the MC58 *nadA* promoter were amplified using Nad-N2/gpr-R and Nad-N5/Nad-Sp primer pairs respectively, to perform experiments on single NadR binding sites of *nadA*. Two pmoles of each fragment were then radioactively labeled at their 5' ends with 30 µCi of (γ-^{32}P)-ATP (6000 Ci/mmol; NEN) using 10 U of T4 polynucleotide kinase (New England Biolabs) and used at the final concentration of 1.6 nM. The unincorporated radioactive nucleotides were removed using TE-10 chromaspin columns (Clontech).

For each binding reaction, 40 fmoles of labeled probe were incubated with recombinant NadR wild type or NadR mutant proteins, suspended in Protein Solution (5 mM Tris-HCl pH 8, 60 mM NaCl), in 25 µl final volume of Gelshift Binding Buffer (40 mM Tris-HCl pH 8, 5 mM MgCl2, 50 mM KCl, 0.05% NP40, 10% glycerol) with 30 nM salmon sperm DNA as non-specific competitor. for 15 min at room temperature, and run on 6% native polyacrylamide gels buffered with 0.5 x TBE, at 100 Volts for 90-180 min at 4°C. When indicated, 1, 2, 5 or 10 mM 4-HPA was added to 100 ng of recombinant NadR WT or NadR mutant proteins in the binding reaction, to test the proteins responses to 4-HPA. Gels were dried and exposed to autoradiographic films at –80°C. To assess the specificity of binding of NadR on the DNA labeled probes, 1.6 nM, 8 nM and 40 nM (1-, 5- or 25-fold

respect to the probe) of either salmon sperm DNA or cold DNA probes were added as non-specific or specific competitors, respectively.

EMSA bands radioactivity was quantified using a phosphorimager and the Image Quant software (Molecular Dynamics).

4.6 DNAse I footprint

The *nadR*, *putA* and *mafA1* promoter regions were amplified with the primer couples P1843-F2/P1843-sR2, P401-F3/P401-R, P375-F/P375-652R, respectively. The PCR products were purified and cloned into pGEMT vector (Promega) as 258, 280 and 267 bp fragments generating pGEMT-P1843, pGEMT-P401 and pGEMT-P375. 2 pmols of each plasmid were 5' end-labeled by T4 polynucleotide kinase with [γ-32P]-ATP after digestion at either the NcoI or SpeI site of the pGEMT polylinker. Following a second digestion with either SpeI or NcoI respectively, the labeled probes were separated from the linearised vector by polyacrylamide gel electrophoresis (PAGE) as described previously [141]. DNA-protein binding reactions were carried out for 15 minutes at RT in footprint buffer (10 mM Tris HCl pH 8, 50 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$ 10% glycerol and 0.05% NP40) containing 40 fmol of labeled probe, 200 ng of sonicated salmon sperm as the non-specific competitor and nM concentrations of NadR purified protein as indicated in the figures. Samples were then treated with 0.03 U of DNase I (Roche) for 1 minute at RT. DNase I digestions were stopped, samples were purified, loaded and run on 8M urea-6% polyacrilamide gels as described [285]. A G+A sequence reaction was performed [286] for each probe and run in parallel to the fooprinting reactions.

4.7 3C mutation scanning

To perform the 3C mutation scanning of the single NadR binding sites on the *nadA* and *mafA1* promoters, DNA oligos corresponding to the forward and reverse strands of either the wild type NadR-protected sequences from DNAI footprints or to mutated sequences, in which sequential triplicate nucleotides were substituted with CCC, were ordered from Sigma. 100 pmols of each oligonucleotide pair were annealed in annealing buffer (10 mM Tris HCl pH 8, 50 mM NaCl and 1 mM EDTA) by using a standard thermal cycler. The mix was heated to 95°C and left at this temperature for 5 minutes, followed by ramp cooling to 25°C over a period of 45 minutes. The annealed oligos were stored at -20°C, 5' end labeled and submitted to EMSA analysis as described in the previous section.

4.8 RNA samples preparation

Bacterial cultures were grown in GC liquid medium to an OD_{600} of 0.5 and then added to the same volume of frozen media to bring the temperature immediately to 4° C. Total RNA was isolated using an RNeasy kit (Qiagen) as described by the manufacturer. RNA pools for microarray experiments were prepared from 3 independent cultures of bacteria. Total RNA was extracted separately from each bacterial pellet and 15 µg of each preparation were pooled together. Three independent pools were prepared for each condition.

4.9 Microarray analyses

DNA microarray analysis was performed using an Agilent custom-designed oligonucleotide array as previously described [252]. cDNA probes were prepared from RNA pools (5 µg) obtained from MC58 wildtype and MC-Δ1843 null mutant cells (see above) and hybridized as described by Fantappiè and colleagues [252]. Three hybridizations were performed using cDNA probes from three independent MC58 and MC-Δ1843 pools, respectively, and the corresponding dyeswap experiments (with MC58 and MC-Δ1843 pools inversely labeled with the Cy3 and Cy5 fluorophores) were also performed. Differentially expressed genes were assessed by grouping all log2 ratio of the Cy5 and Cy3 values corresponding to each gene, within experimental replicas and spot replicas, and comparing them against the zero value by Student's t-test statistics (one tail).

The array layout was submitted to the EBI ArrayExpress and it is available with the identifier A-MEXP-1967. The entire set of supporting microarray data has been deposited in the ArrayExpress public database under the accession number E-MTAB-803.

4.10 Quantitative Real Time PCR (qRT-PCR)

Approximately 4 µg of the total RNA preparation was treated with RQ1 RNase-free DNase (Promega). RNA was then reverse transcribed using random hexamer primers and Moloney murine leukemia virus reverse transcriptase (Promega) as recommended by the manufacturer. For negative controls, all RNA samples were also incubated without reverse transcriptase. Primer pairs for each target gene were designed and optimized and are listed in Appendix Table 3 named with the number according to their NMB annotation

and rt for RT-PCR. Primers 16S_F and 16S_R were used for the 16S rRNA normalization control (Appendix Table 3). All RT-PCRs were performed in triplicate using a 25 µl mixture containing cDNA (5 µl of a 1/5 dilution), 1x brilliant SYBR green quantitative PCR master mixture (Stratagene), and approximately 5 pmol of each primer. Amplification and detection of specific products were performed with an Mx3000 real-time PCR system (Stratagene) using the following procedure: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s and then a dissociation curve analysis. The 16S rRNA gene was used as the endogenous reference control, and relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ relative quantification method.

4.11 Human saliva samples

Human saliva was obtained from healthy non-smoking donors in the morning at least ten hours after eating and after rinsing the mouth with water. In order to minimize the degradation of the proteins the sample was kept on ice during the collection process. Immediately after collection, the sample of human saliva was stored at -20°C.

4.12 Human serum samples

Healthy human volunteers were immunized under informed consent with the experimental serogroup B vaccine containing 50 µg each of GNA2091-fHbp, NHBA-GNA1030, and NadA and 25 µg of Outer Membrane Vesicles from the New Zealand strain NZ98/254, adsorbed to aluminium hydroxide. Serum samples before and after immunization were obtained from the following clinical trials:

Study 1 was a clinical trial conducted in healthy adults, laboratory workers. Pooled sera were derived from 23 subjects before and after 3 doses of 4CMenB at 0, 2 and 6 months.
Study 2 was a clinical study evaluating the safety, immunogenicity and lot consistency of 4CMenB administered to infants at 2, 4 and 6 months of age. Extensions of this clinical study investigated a fourth (booster) dose at 12 months of age. Pooled sera were derived from 107 infants at 7 months of age who received the primary series of 3 doses of routine vaccine at 2, 4 and 6 month of age and from 141 infants who received the primary series of 3 doses of 4CMenB at 2, 4 and 6 month of age plus a booster in the second year of life.
Study 3 was a clinical study evaluating the safety, tolerability and immunogenicity of 4CMenB administered to infants at 2, 4 and 6 months of age. Extensions of this clinical study investigated a fourth (booster) dose at 12, 18 or 24 months of age. Pooled sera were derived from 109 infants at 5 months of age who received the primary series of 3 doses of routine vaccine at 2, 3 and 4 month of age and from 69 infants who received the primary series of 3 doses of 4CMenB at 2, 4 and 6 months of age plus a booster in the second year of life.

4.13 Immunization of mice

To prepare antisera, 20 µg of individual antigens NadA, NHBA-GNA1030, GNA2091-fHbp or a combination of 20 µg of each of NHBA-GNA1030, GNA2091-fHbp and NadA with or without 10 µg of deoxycholate-extracted OMV derived from the strain NZ98/254 were used to immunize 6-week-old CD1 female mice (Charles River). Five to ten mice per group were used. The antigens were administered intraperitoneally (i.p.), together with aluminium hydroxide (3 mg/ml) on days 0, 21 and 35.

4.14 Serum bactericidal assay (SBA)

Serum bactericidal antibody activity against *Neisseria meningitidis* strains with mice antisera was evaluated as previously described [287] with pooled baby rabbit serum used as the complement source (rSBA). Serum bactericidal antibody assays with human complement (hSBA) were performed as described by Borrow et al. with minor modifications [287]. MenB bacteria were subcultured overnight on Chocolate Agar and resuspended in Mueller Hinton medium (MHM) to an OD_{600} of 0.25 before use in the assay. Serum bactericidal titers were determined as the last dilution that resulted in at least a 50% reduction in colony forming units (CFU) relative to the number of CFU present at the beginning of the bactericidal reaction (i.e. 50% of T0). Human plasma obtained from volunteer donors after informed consent was selected for use as complement source with a particular MenB strain only if it did not significantly reduce CFU of that strain relative to T0 when added to the assay at a final concentration of 50%. The final assay mixture contained 25% human plasma complement. When required, 4-HPA and 3Cl4-HPA in aqueous solution were added to chocolate agar at a final concentration of 5 mM.

4.15 Meningococcal Antigen Typing System (MATS) ELISA

The MATS assay was been performed as previously described [243]. Briefly, bacteria are grown overnight on Chocolate Agar plates (Biomerieux) ± 4-HPA and 3Cl4-HPA supplementation. Bacterial suspensions are prepared from overnight cultures, single colonies being collected and suspended to an OD_{600} = 0.4, using Ultrospec 10 Classic (Amersham Biosciences). Bacterial suspensions are lysed with a detergent (EmpigenBB

5%, Sigma) added to 1/10 volume, followed by incubationfor 1 hour at 45°C in a shaking water bath. Samples are added to ELISA microwell plates, which were previously coated with polyclonal rabbit antibody against NadA (961c) to capture the antigen from the lysate, and diluted at 2-fold step dilution. Captured antigen is then detected with a biotin-labeled rabbit antibody (Biotin-Ab) against the antigen followed by HRP-labeled streptavidin. The signal is provided by reduction of o-phenylenediamine substrate (OPD, Sigma). Each plate contains 1 reference strain and 6 sample strains: 5/99 is used as the reference strain for NadA. Relative Potency is a mathematically-determined quantity obtained by comparing the serial dilution curves of the test sample and the reference strain. Raw data reduction and analysis is performed by StatLIA (Brendan Technologies).

4.16 Passive protection and *in vivo* imaging in infant rats.

The ability of anti-NadA antibodies to confer passive protection against *N. meningitidis* bacteremia was tested in infant rats challenged intraperitoneally (i.p.). *In vivo* imaging of the bioluminescence of the P*nadA-lux* reporter and control strains was monitored in infant rats infected i.p. The day before the challenge/infection, freshly thawed bacteria were inoculated onto chocolate agar and were grown overnight at 37°C in 5% CO_2. On the morning of the challenge/infection, several colonies were inoculated in MHM supplemented with 0.25% (wt/vol) glucose to a starting OD_{600} of~0.05. After 1.5 h growth at 37°C in 5% CO_2, the bacterial suspension reached an OD_{600} of ~0.25 and was diluted in PBS to obtain 10^5-10^7 CFU/ml. 5–7-day-old pups from litters of out-bred Wistar rats (Charles River) were numbered. The protection and challenge were performed as follows: Groups of 3–19 animals were treated i.p. at time 0 with 100 µl doses of different dilutions

of test or control antisera. Three hours later, the animals were challenged i.p. with a 100μl dose of 10^5 CFU of *N. meningitidis* strain NGP165. Eighteen hours after the bacterial challenge, blood samples were obtained by cheek puncture with a syringe containing 25U of heparin without preservative (American Pharmaceutical Partnersand CFUs were measured. Rats were considered infected when >10 CFUs were counted on plates carrying 100 μl of blood. Counts above the threshold were verified for positivity by examining plates carrying 10- and 100-fold dilutions of blood. For *in vivo* imaging of bioluminescent reporters, groups of 5 animals were inoculated i.p. at time 0 with 100 μl doses of 10^4 CFU of the 2996-*lux* or 2996-P*nad*-*lux* strains. Rats were then anesthetized using a constant flow of 2.5% isoflurane mixed with oxygen. Bioluminescence measurements of ventral views of each group of rats were taken of at time 0, 3 and 24 hours, using a an IVIS 100 system (Xenogen Corp., Alameda, CA) according to instructions from the manufacturer. Analysis and acquisition were performed using Living Image 3.1 software (Xenogen Corp.). Quantifying was performed using the photons per second emitted by each rat. 2 rats infected with the 2996 wild type strain under the same conditions of acquisition were used for subtracting the background. Twenty four hours after infection, blood samples were obtained and CFU counts measured.

5 APPENDIX

5.1 Table 1 - Strains used in this study

Strains	Relevant characteristics	Reference
N.meningitidis		
MC58	Clinical isolate, sequenced strain containing 9 TAAA tetranucleotide repeats in the *nadA* promoter, CC32	[62]
5/99	Clinical isolate containing 8 TAAA tetranucleotide repeats in the *nadA* promoter, CC8	NIPH[1]
961-5945	Clinical isolate containing 12 TAAA tetranucleotide repeats in the *nadA* promoter, CC8	[203]
LNP17094	Clinical isolate containing 12 TAAA tetranucleotide repeats in the *nadA* promoter, CC32	[243]
B3937	Clinical isolate containing 12 TAAA tetranucleotide repeats in the *nadA* promoter, CC18	[243]
M10574	Clinical isolate containing 6 TAAA tetranucleotide repeats in the *nadA* promoter, CC32	CDC[2]
M14933	Clinical isolate containing 6 TAAA tetranucleotide repeats in the *nadA* promoter, CC32	CDC[2]
NGP165	Clinical isolate containing 9 TAAA tetranucleotide repeats in the *nadA* promoter, CC11	[243]
2996	Clinical isolate, CC8	[243]
NM036	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM037	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM066	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM067	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM069	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM081	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM088	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM100	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM119	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM145	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM154	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM156	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM188	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
NM191	Strain carrying the *nadA* gene from a collection of 31 clinical isolates from children ≤ 4 years of age, convalescing after meningococcal disease	[205]
MC-Δ1843::Cm^R	NadR null mutant in MC58 strain, generated with the pΔ1843ko::Cm^R plasmid, Cm^R	[141]

MC-Δ1843::Kan^R	NadR null mutant in MC58 strain, generated with the pΔ1843ko::Kan^R plasmid, Kan^R	This study
5/99-Δ1843	NadR null mutant in 5/99 strain, generated with the pΔ1843ko::Cm^R plasmid, Cm^R	[141]
961-Δ1843	NadR null mutant in 961-5945 strain, generated with the pΔ1843ko::Cm^R plasmid, Cm^R	[141]
LNP-Δ1843	NadR null mutant in LNP17094 strain, generated with the pΔ1843ko::Cm^R plasmid, Cm^R	This study
B39-Δ1843	NadR null mutant in B3937 strain, generated with the pΔ1843ko::Cm^R plasmid, Cm^R	This study
M10-Δ1843	NadR null mutant in M10574 strain, generated with the pΔ1843ko::Cm^R plasmid, Cm^R	This study
M14-Δ1843	NadR null mutant in M14933 strain, generated with the pΔ1843ko::Cm^R plasmid, Cm^R	This study
MC-Δ1843_C	MC-Δ1843::Cm^R derivative, NadR complemented strain expressing the *nadR* wild type gene under the control of the inducible P*tac* promoter between the converging open reading frames NMB1428 and NMB1429, Cm^R and Ery^R	This study
MC-Δ1843_CH7A	MC-Δ1843::Cm^R derivative, NadR complemented strain expressing the *nadR* H7A mutant gene, Cm^R and Ery^R	This study
MC-Δ1843_CS9A	MC-Δ1843::Cm^R derivative, NadR complemented strain expressing the *nadR* S9A mutant gene, Cm^R and Ery^R	This study
MC-Δ1843_CN11A	MC-Δ1843::Cm^R derivative, NadR complemented strain expressing the *nadR* N11A mutant gene, Cm^R and Ery^R	This study
MC-Δ1843_CR18A	MC-Δ1843::Cm^R derivative, NadR complemented strain expressing the *nadR* R18A mutant gene, Cm^R and Ery^R	This study
MC-Δ1843_CF25A	MC-Δ1843::Cm^R derivative, NadR complemented strain expressing the *nadR* F25A mutant gene, Cm^R and Ery^R	This study
MC-Δ1843_CW39A	MC-Δ1843::Cm^R derivative, NadR complemented strain expressing the *nadR* W39A mutant gene, Cm^R and Ery^R	This study
MC-Δ1843_CR40A	MC-Δ1843::Cm^R derivative, NadR complemented strain expressing the *nadR* R40A mutant gene, Cm^R and Ery^R	This study
MC-Δ1843_CY115A	MC-Δ1843::Cm^R derivative, NadR complemented strain expressing the *nadR* Y115A mutant gene, Cm^R and Ery^R	This study
MC58-*lux*	MC58 derivative, carrying the promoterless *lux* operon, Cm^R	This study
MC58-P*nadA*-*lux*	MC58 derivative, carrying the *lux* operon under the control of the promoter of NadA with 9 TAAA tetranucleotide repeats, Cm^R	This study
MC58-Δ1843-*lux*	MC-Δ1843::Kan^R derivative, carrying the promoterless *lux* operon, Kan^R and Cm^R	This study
MC58-Δ1843-P*nadA*-*lux*	MC-Δ1843::Kan^R derivative, carrying the *lux* operon under the control of the promoter of NadA with 9 TAAA tetranucleotide repeats, Kan^R and Cm^R	This study
2996-*lux*	2996 derivative, carrying the promoterless *lux* operon, Cm^R	This study
2996-P*nadA*-*lux*	2996 derivative, carrying the *lux* operon under the control of the promoter of NadA with 9 TAAA tetranucleotide repeats, Cm^R	This study
E.coli		
DH5-α	supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1	[288]
BL21(DE3)	hsdS gal (λcIts857 ind1 Sam7 nin-5 lacUV5-T7 gene 1)	[281]
BL21(DE3)-1843R18A	BL21(DE3) derivative strain carrying the pET15-1843R18A plasmid for expression and purification of the mutated NadR, Amp^R	This study
BL21(DE3)-1843RW39A	BL21(DE3) derivative strain carrying the pET15-1843 W39A plasmid for expression and purification of the mutated NadR, Amp^R	This study
BL21(DE3)-1843R40A	BL21(DE3) derivative strain carrying the pET15-1843 R40A plasmid for expression and purification of the mutated NadR, Amp^R	This study
BL21(DE3)-1843Y115A	BL21(DE3) derivative strain carrying the pET15-1843 Y115A plasmid for expression and purification of the mutated NadR, Amp^R	This study

[1] The Norwegian Institute of Public Health
[2] Centers for Disease Control and Prevention

5.2 Table 2 - Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pET15b	Expression vector for N-terminal His tagged proteins, Amp^R	Invitrogen
pET15-1843	pET15b derivative for expression of recombinant 1843 protein, Amp^R	[141]
pET15-1843R18A	pET15-1843 derivative for expression of recombinant 1843-R18A mutant protein, Amp^R	This study
pET15-1843W39A	pET15-1843 derivative for expression of recombinant 1843-W39A mutant protein, Amp^R	This study
pET15-1843R40A	pET15-1843 derivative for expression of recombinant 1843-R40A mutant protein, Amp^R	This study
pET15-1843Y115A	pET15-1843 derivative for expression of recombinant 1843-Y115A mutant protein, Amp^R	This study
pGEM-T	*E.coli* cloning vector, Amp^R	Promega
pΔ1843ko::Cm^R	Construct for generating knockout of the NMB1843 gene, Cm^R	[141]
pΔ1843ko::Kan^R	Construct for generating knockout of the NMB1843 gene, Kan^R	This study
pSLComCmr	Plasmid consisting of the chloramphenicol resistance gene flanked by upstream and downstream regions for allelic replacement at a chromosomal location between ORFs NMB1428 and NMB1429, Cm^R	[118]
pComEryPind-NadR	Plasmid for complementation of the NadR null mutant, derivative of pSLComCmr containing a copy of the *nadr* gene under the control of the P_{tac} promoter, Ery^R	This study
pComEry-1843H7A	Plasmid for complementation of the NadR null mutant with the H7A mutant form of *nadR*, derivative of pComEry-NadR, Ery^R	This study
pComEry-1843S9A	Plasmid for complementation of the NadR null mutant with the S9A mutant form of *nadR*, derivative of pComEry-NadR, Ery^R	This study
pComEry-1843N11A	Plasmid for complementation of the NadR null mutant with the N11A mutant form of *nadR*, derivative of pComEry-NadR, Ery^R	This study
pComEry-184 R18A	Plasmid for complementation of the NadR null mutant with the R18A mutant form of *nadR*, derivative of pComEry-NadR, Ery^R	This study
pComEry-1843F25A	Plasmid for complementation of the NadR null mutant with the F25A mutant form of *nadR*, derivative of pComEry-NadR, Ery^R	This study
pComEry-1843W39A	Plasmid for complementation of the NadR null mutant with the W39A mutant form of *nadR*, derivative of pComEry-NadR, Ery^R	This study
pComEry-1843R40A	Plasmid for complementation of the NadR null mutant with the R40A mutant form of *nadR*, derivative of pComEry-NadR, Ery^R	This study
pComEry-1843Y115A	Plasmid for complementation of the NadR null mutant with the Y115A mutant form of *nadR*, derivative of pComEry-NadR, Ery^R	This study
pSB1075	N-acyl homoserine lactone biosensor based on *lasR::lasI'luxCDABE*, Amp^R	[283]
pBleuskcript II	*E. coli* cloning and expression vector, Amp^R	[9]
pSL-*furlacZ*	Plasmid containing upstream and downstream regions for allelic replacement at a chromosomal location between ORFs NMB1074 and NMB1075, Ery^R	[94]
pSL-*lux*-Fla	pSL-*furlacZ* derivative with the promoterless *luxCDABE* operon and a chloramphenicol cassette, Cm^R	This study
pSL-P*nadA*-*lux*	pSL-*lux*-Fla derivative with the *luxCDABE* operon under the control of the promoter of *nadA* and a chloramphenicol cassette, Cm^R	This study

5.3 Table 3 - Oligonucleotides used in this study

Name	Sequence[1]	Site
1843-F	attcacatATGCCTACCCAATCAAAACATGCG	NdeI
1843-R2	attcatgcatCGGCGTATTACGAGTTCAACGCATCCTCG	NsiI
EryXbaF	attcgtctagaGCAAACTTAAGAGTGTGTTGATAG	XbaI
EryBamR	atatatggatccGGGACCTCTTTAGCTTCTTGG	BamHI
NadluxF2	attcactcgaggctagcTAAGACACGACACCGGCAGAATTG	XhoI-NheI
NadluxR2	attcaggtaccTACGCTCATTACCTTTGTGAGTGG	KpnI
119rt-R	AGTGTTGCCCTGTGTAGCAG	
120rt-F	GCATGGATTATAACCTATCGATCTAA	
120rt-R	TACGCTTGCTGTTCCCTGT	
207rt-F	TGACCAAATTCGACACCGT	
207rt-R	ATCGACACCGAGTTCTTTCC	
401rt-L	AGGTATCGGTTTCGTTGTCC	
401rt-R	TTGATTTCGCTGTCCCAATA	
652-375rt-F	CGAATATTTCGCCGTTGAC	
652-375rt-R	AAAGGGCGTATTGTTCTTGG	
702rt-F	GATTTGCTGCTGTCGGACTA	
702rt-R	GCCTATCCCGTTGGATAATG	
865rt-F	ACCGCTGGTGGTATGATAGG	
865rt-R	GCACCATTTGCACGAATAAC	
955rt-F	TGTGCCGGTCAGGTTTACTA	
955rt-R	GTTCGGATATTTCGCCAGTT	
978rt-F	AAACACAAGCTCAACGCACT	
978rt-R	GGGCAATCAGGGTCATAATC	
980rt-F	TTTGAAACCGTTGTCGAAAG	
980rt-R	GGCGCGTTGACCTTATAAAT	
1277rt-F	AGACGCAGGAGCAGGATATT	
1277rt-R	CCGTATTCTTCCGAAAGCTC	
1476rt-F	ACGTTGCCATTTACAACGAA	
1476rt-R	GTTCGGCGTTGGTAATTTCT	
1478rt-F	CATCGGCAAACTGGTTCA	
1478rt-R	CTCAAATGGTCGCGGTAGTA	
1609rt-F	TACTACACCGGATTGTCCGA	
1609rt-R	CTTCTTGATCGGCAACTTCA	
1841rt-F	CCTTTGACCGATACCACTCC	
1841rt-R	ATGACGATTTCGGTAAACCC	
1842rt-F	TTTCAAACTTCGTTCCGTGA	
1842rt-R	CCGTCATTGTAATGCCGTAA	
1843rt-F	AGGGCGAGAAGCTGTATGAG	
1843rt-R	CAGGTCTTTAAGCAGCAGCA	
1844rt-F	ATGCGGTTTATAGCGTATTG	
1844rt-R	TTAAGTCTCCAAGTTATCG	
1994rt-F	GCTGGCACAGCTAATACTGC	
1994rt-R	TCAGCTTTGTTCGTAGCGAT	
2099rt-F	ACCTGCCTTATATCGAACCG	
2099rt-R	AGGTTCAAATCATGCACACG	
16S_F	ACGGAGGGTGCGAGCGTTAATC	
16S_R	CTGCCTTCGCCTTCGGTATTCCT	
P207-F2	CTCAAACAATACAAAGCCAAACAGG	
P207-R	GCCCATGGTTTGTTCCTTTGTTGAGGG	
P375-F	CCGCAATGGGTGGAAGCCGCCGC	
P652-F	ACAGTCAAAATGCCGTCTGAAAGCC	

P375-652R	GGAGGAGCAGGGTTTTCATAGCGGGG	
P401-F3	GCCGACGCAGATTACCGCGCC	
P401-R	GCCGGAAATGCAAAATGAAACATTTTTTGG	
P0430-F	CAAAGGAACATTACTATGAAACC	
P0430-R	GTGTTGACTCATCATATTTCTCC	
P0535-F	CGGGCATACGACATTCTTTCCGC	
P0535-R	GACATTTCTTAACGGCAATGC	
P0702-F	CCCTGAGTCCTAGATTCCCGC	
P0702-R	CCGAAAAACCGTCATAACAAGATTTG	
P0955-F2	TACCGGTTCGGAACGCCGCG	
P0955-R	CGTCCATCATGGCGTGCGC	
P0978-F2	CGGCACACGCAACCGGCAATGCGGCGC	
P0978-R	CGAGTCCTGAAGACATAGAAATTCTCCG	
P0980-F2	CGGCAAAGAATGTTACGGCGGGCGG	
P0980-R	CGCGTGGGATACCGATTTTCATCTCTG	
P1205-F	CCAACGAAGAACATATAGACTGGCTGG	
P1205-R	CAAGCACCCTGAATTTCATATCGG	
P1277-F	CCGGCGTTAAACGCCCCG	
P1277-R	CAGACAGGGACAAACCTTCTCACTCC	
P1476-F2	GCAAATCCCGGCGCCGTTCC	
P1476-R	GGGCTTCAGACATTTTGCTTCC	
P1478-F2	CAAGCCGCACGGAATCCGTCTG	
P1478-R	CGATGGCTGCATTCATAATCCGG	
P1609-F	CCTATCAGCTCCAGCAGATGC	
P1609-R	GCTCATCGGTGATTCCTCGG	
P1843-F2	GAGCCGACGCGCCTGCCGATG	
P1843-sR2	GGTAGGCATTGTTTAAGTCTC	
1844-F	GGGAAAGAGCCGACGCGCCTG	
1844-R	GCTATAAACCGCATCGGACGACTGG	
P2099-F	CCGCCTGTTCCTGCTGCC	
P2099-R	CCTCGTTCCTCATTTCAGACGGCC	
Nad-N1	attcagatgcatGACGTCGACGTCCTCGATTACGAAGGC	NsiI
Nad-B1	attcaggatcctacGCTCATTACCTTTGTGAGTGG	BamHI
Nad-N2	attcagatgcatTAAGACACGACACCGGCAGAATTG	NsiI
gpr-R	gattagcatgcCGGCATTAATATCTGTTAATATGTGC	SphI
Nad-N5	attcagatgcatCTTTAATATGTAAACAAACTTGGTGG	NsiI
Nad-Sp	attcagcatgctacGCTCATTACCTTTGTGAGTGG	SphI
H7A-F	TGCCTACCCAATCAAAAgcgGCGTCTATCAATATCGG	
H7A-R	CCGATATTGATAGACGCcgcTTTTGATTGGGTAGGCA	
S9A-F	CCCAATCAAAACATGCGgCgATCAATATCGGTCTGATAC	
S9A-R	GTATCAGACCGATATTGATcGcCGCATGTTTTGATTGGG	
N11A-F	CAAAACATGCGTCTATCgcgATCGGTCTGATACAGGC	
N11A-R	GCCTGTATCAGACCGATcgcGATAGACGCATGTTTTG	
R18A-F	CGGTCTGATACAGGCAgcGGAAGCCCTG	
R18A-R	CAGGGCTTCCgcTGCCTGTATCAGACCG	
F25A-F	GAAGCCCTGATGACCCAAgcCAGGCCTATTCTGAATCAG	
F25A-R	CTGATTCAGAATAGGCCTGgcTTGGGTCATCAGGGCTTC	
W39A-F	CCGATCAGCAAgcGCGGATTATCCGTC	
W39A-R	GACGGATAATCCGCgcTTGCTGATCGG	
R40A-F	CCGATCAGCAATGGgcGATTATCCGTC	
R40A-R	GACGGATAATCgcCCATTGCTGATCGG	
Y115A-F	GTGGACGAACGCgcCGACGCTATCGAGG	
Y115A-R	CCTCGATAGCGTCGgcGCGTTCGTCCAC	
NadA OP1 F	GCATAATATGCACATATTAACA	
NadA OP1 R	TGTTAATATGTGCATATTATGC	

NadA OP2 F	TATATACTTTAATATGTAAACA	
NadA OP2 R	TGTTTACATATTAAAGTATATA	
MafA OP F	ACTCAAAATCCGTTCAACATCAAACAAA	
MafA OP R	TTTGTTTGATGTTGAACGGATTTTGAGT	
NadA1mut1F	cccTAATATGCACATATTAACA	
NadA1mut1R	TGTTAATATGTGCATATTAggg	
NadA1mut2F	GCAcccTATGCACATATTAACA	
NadA1mut2R	TGTTAATATGTGCATAgggTGC	
NadA1mut3F	GCATAAcccGCACATATTAACA	
NadA1mut3R	TGTTAATATGTGCgggTTATGC	
NadA1mut4F	GCATAATATcccCATATTAACA	
NadA1mut4R	TGTTAATATGgggATATTATGC	
NadA1mut5F	GCATAATATGCAcccATTAACA	
NadA1mut5R	TGTTAATgggTGCATATTATGC	
NadA1mut6F	GCATAATATGCACATcccAACA	
NadA1mut6R	TGTTgggATGTGCATATTATGC	
NadA1mut7F	GCATAATATGCACATATTcccA	
NadA1mut7R	TgggAATATGTGCATATTATGC	
MafAmut1F	cccCAAAATCCGTTCAACATCAAACAAA	
MafAmut1R	TTTGTTTGATGTTGAACGGATTTTGggg	
MafAmut2F	ACTcccAATCCGTTCAACATCAAACAAA	
MafAmut2R	TTTGTTTGATGTTGAACGGATTgggAGT	
MafAmut3F	ACTCAAcccCCGTTCAACATCAAACAAA	
MafAmut3R	TTTGTTTGATGTTGAACGGgggTTGAGT	
MafAmut4F	ACTCAAAATcccTTCAACATCAAACAAA	
MafAmut4R	TTTGTTTGATGTTGAAgggATTTTGAGT	
MafAmut5F	ACTCAAAATCCGcccAACATCAAACAAA	
MafAmut5R	TTTGTTTGATGTTgggCGGATTTTGAGT	
MafAmut6F	ACTCAAAATCCGTTCcccATCAAACAAA	
MafAmut6R	TTTGTTTGATgggGAACGGATTTTGAGT	
MafAmut7F	ACTCAAAATCCGTTCAACcccAAACAAA	
MafAmut7R	TTTGTTTgggGTTGAACGGATTTTGAGT	
MafAmut8F	ACTCAAAATCCGTTCAACATCcccCAAA	
MafAmut8R	TTTGgggGATGTTGAACGGATTTTGAGT	
MafAmut9F	ACTCAAAATCCGTTCAACATCAAAcccA	
MafAmut9R	TgggTTTGATGTTGAACGGATTTTGAGT	
NadA2mut1F	cccATACTTTAATATGTAAACA	
NadA2mut1R	TGTTTACATATTAAAGTATggg	
NadA2mut2F	TATcccCTTTAATATGTAAACA	
NadA2mut2R	TGTTTACATATTAAAGgggATA	
NadA2mut3F	TATATAcccTAATATGTAAACA	
NadA2mut3R	TGTTTACATATTAgggTATATA	
NadA2mut4F	TATATACTTcccTATGTAAACA	
NadA2mut4R	TGTTTACATAgggAAGTATATA	
NadA2mut5F	TATATACTTTAAcccGTAAACA	
NadA2mut5R	TGTTTACgggTTAAAGTATATA	
NadA2mut6F	TATATACTTTAATATcccAACA	
NadA2mut6R	TGTTgggATATTAAAGTATATA	
NadA2mut7F	TATATACTTTAATATGTAcccA	
NadA2mut7R	TgggTACATATTAAAGTATATA	

[1] Capital letters indicate *N. meningitidis* derived sequences, small letters indicate sequences added for cloning purpose, underlined letters indicate the site included in the oligonucleotide.

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