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A novel phase variation mechanism in the meningococcus driven by a ligand-responsive repressor and differential spacing of distal promoter elements

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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 in seguito a stess ossidativo, ho caratterizzato un circuito di regolazione che coinvolge un piccolo RNA non codificante (sRNA) e lo chaperone Hfq, e piú in dettaglio ho approfondito la complessa regolazione trascrizionale di NadA, argomento quest'ultimo del lavoro di tesi di seguito presentato.

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

Ieva R, Roncarati D, **Metruccio MM**, Seib KL, Scarlato V, Delany I. "OxyR tightly regulates catalase expression in *Neisseria meningitidis* through both repressive and activation mechanisms." *Mol Microbiol. 2008 Dec;70(5):1152-65.*

Metruccio MM, Fantappiè L, Serruto D, Muzzi A, Roncarati D, Donati C, Scarlato V, Delany I. "The Hfq-dependent small noncoding RNA NrrF directly mediates Fur-dependent positive regulation of succinate dehydrogenase in *Neisseria meningitidis." J Bacteriol. 2009 Feb;191(4):1330-42. Epub 2008 Dec 5.*

Fantappiè L, **Metruccio MM**, Seib KL, Oriente F, Cartocci E, Ferlicca F, Giuliani MM, Scarlato V, Delany I. **"**The RNA chaperone Hfq is involved in the stress response and virulence in *Neisseria meningitidis* and is a pleiotropic regulator of protein expression." *Infect Immun. 2009 May;77(5):1842-53. Epub 2009 Feb 17.*

Metruccio MM, Pigozzi E, Roncarati D, Berlanda-Scorza F, Norais N, Hill S, Scarlato V, Delany I. "A novel phase variation mechanism in meningococcus driven by a ligand responsive repressor and differential spacing of distal promoter elements*."* PLoS Pathog. 2009 Dec;5(12): e1000710. Epub 2009 Dec 24.

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Riassunto

Il rapido adattamento all´ambiente circostante costituisce uno dei requisiti fondamentali per i batteri patogeni. La capacitá di alcuni batteri di esprimere determinati geni in maniera casuale (variazione di fase), grazie al cambiamento nella lunghezza di sequenze semplici ripetute (SSR) durante la replicazione, conferisce alla popolazione batterica una varietá che le permette un piú rapido adattamento ai cambiamenti dell´ambiente circostante. In questo lavoro si mostra un nuovo meccanismo alla base della "variazione di fase" di NadA, un invasina e adesina di *Neisseria meningitidis.* Il repressore NadR contatta due operatori sul DNA posizionati alle estremitá del tratto di sequenze ripetute sul promotore di *nadA,* e contribuisce al diverso livello di espressione in promotori con diverso numero di ripetizioni probabilmente a causa della variazione della distanza relativa tra i due operatori. Qui si dimostra inoltre che IHF lega tra questi operatori e, probabilmente tramite la formazione di una piega del DNA, favorirebbe l´interazione di NadR legato all´operatore distale con quello sovrapposto al promotore. L´acido 4-idrossifenilacetico, un metabolita del catabolismo degli aminoacidi aromatici che viene secreto nella saliva, induce l´espressione di NadA inibendo il legame del repressore al DNA. Tra i promotori con diverso numero di ripetizioni sono osservabili solo minime differenze nei livelli di trascrizione di *nadA* in assenza di NadR, queste differenze sono probabilmente dovute ad un diverso legame della RNA polimerasi sui diversi promotori, che porta ad un´alterata attivitá trascrizionale. Questi risultati suggeriscono che l´espressione di NadA si trova sotto il controllo di eventi sia casuali che finemente regolati in base all´ambiente, entrambi mediati dal repressore NadR, e che questa importante adesina potrebbe essere indotta durante la colonizzazione dell´orofaringe, dove esplica un ruolo importante nell´adesione e invasione della mucosa. In conclusione, la presenza di semplici sequenze ripetute nei promotori, puó essere una

strategia sfruttata da alcuni batteri patogeni per modificare casualmente il livello di espressione di alcuni geni, pur mantenendo la possibilitá di indurli in presenza del segnale specifico.

Abstract

Phase variable expression, mediated by high frequency reversible changes in the length of simple sequence repeats, facilitates adaptation of bacterial populations to changing environments and is frequently important in bacterial virulence. Here we elucidate a novel phase variable mechanism for NadA expression, an adhesin and invasin of *Neisseria meningitidis*. The NadR repressor protein binds to operators flanking the phase variable tract of the *nadA* promoter gene and contributes to the differential expression levels of phase variant promoters with different numbers of repeats, likely due to different spacing between operators. It is shown that IHF binds between these operators, and may permit looping of the promoter, allowing interaction of NadR at operators located distally or overlapping the promoter. The 4-hydroxyphenylacetic acid, a metabolite of aromatic amino acid catabolism that is secreted in saliva, induces *nadA* expression by inhibiting the DNA binding activity of the NadR repressor. When induced, only minor differences are evident between NadR-independent transcription levels of promoter phase variants, which are likely due to differential RNA polymerase contacts leading to altered promoter activity. These results suggest that NadA expression is under both stochastic and tight environmental-sensing regulatory control, and both regulations are mediated by the NadR repressor that and may be induced during colonization of the oropharynx where it plays a major role in the successful adhesion and invasion of the mucosa. Hence, simple sequence repeats in promoter regions may be a strategy used by host-adapted bacterial pathogens to randomly switch between expression states that may nonetheless still be induced by appropriate niche-specific signals.

Introduction

1.1 Meningococcal disease

Neisseria meningitidis is a strictly human pathogen responsible for meningitis and sepsis, two devastating diseases that can kill children and young adults within hours, despite the availability of effective antibiotics. The reported annual incidence of meningococcal disease varies from 0.5 to 10 per 100,000 persons; however, during epidemics the incidence can rise above 1 per 1,000 (Stephens 2009). The case fatality rate ranges from 5 to 15%, and up to 25% of survivors are left with neurological sequelae (Comanducci, Bambini et al. 2002).

N. meningitidis colonises the upper respiratory tract in about 25% of the human population where it can live as commensal. This carrier state not only provides a reservoir for meningococcal infection but can also contribute to establish host immunity (Stephens 2009). Furthermore, the human host is the only known reservoir for this human-adapted bacterium. For largely unknown reasons, dependent from both host and pathogen, in a small subset of carriers, meningococcus can invade the pharyngeal mucosal epithelium and, in the absence of bactericidal serum activity, disseminate into the bloodstream, causing septicaemia. In a subset of cases, the bacteria can also cross the blood-brain barrier and infect the cerebrospinal fluid, causing meningitis.

1.2 The pathogen

N. meningitidis is a Gram-negative, spherical or kidney-shaped bacillus commonly seen in pairs (diplococcus). It is aerobic, non-motile, non-sporulating, usually encapsulated and piliated (Figure 1). Traditionally, different strains of *N. meningitidis* are classified on the bases of the composition and antigenic property of their capsule. With this

method, 13 serogroups can be distinguish, five of which (A, B, C, W-135 and Y) are responsible for virtually all meningococcal disease. Meningococci are further classified into serotype and serosubtype, based on antigenic differences in their major outer membrane proteins (OMPs), PorA and PorB. However, since this classification is based on variation of few genes that are probably under selective pressure, serotyping is not suitable for modern epidemiology. A genetic typing system based upon polymorphisms in multiple housekeeping genes called Multilocus Sequence Typing (MLST) (Maiden, Bygraves et al. 1998), is now the gold standard for molecular typing and epidemiologic studies, although does not correlate with antigenic composition of the bacteria which remains largely variable among lineages. MLST technique has shown that the majority of disease associated isolates cluster into a minority of sequence type (ST) called hyper invasive lineage (Maiden 2008). Why hyper virulent meningococcal lineages are more pathogenic than others remain still unknown.

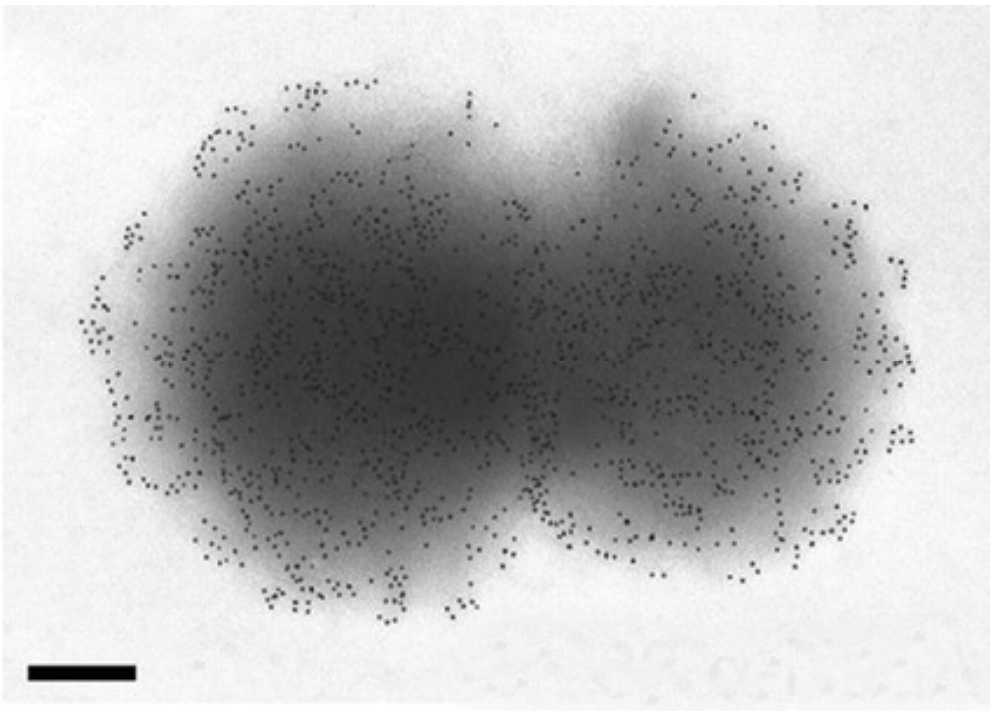


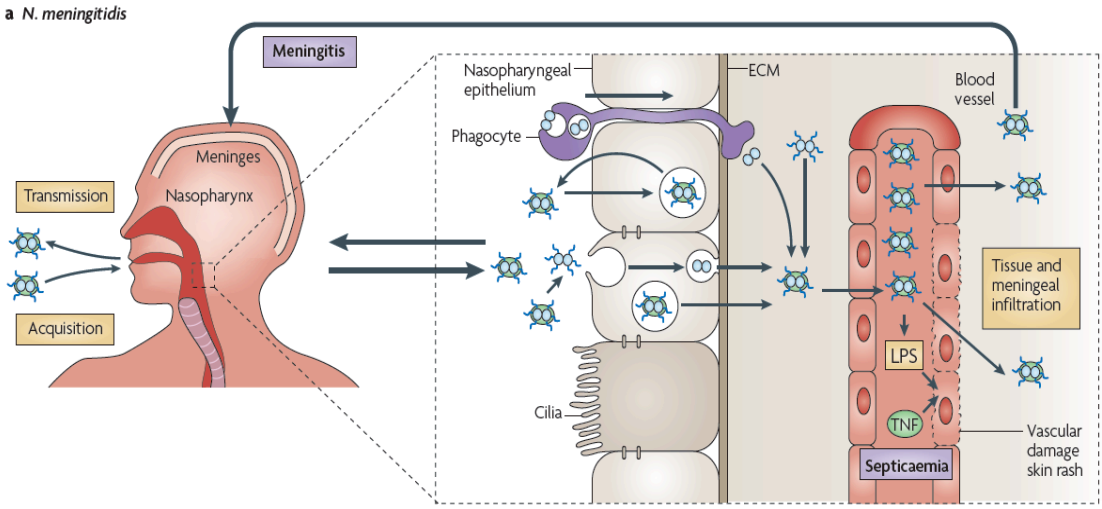
Figure 1: ImmunoGold labelling and transmission electron microscopy of *Neisseria meningitidis* strain. Analysis of the strain was performed with antisera raised against NadA adhesin (Scale bars: 200 nm.) (Pizza, Scarlato et al. 2000).

1.3 Genomes

Up to now seven *N. meningitidis* genomes have been sequenced, four disease associated and three carriage strains These data show that the meningococcal chromosome is between 2.0 and 2.1 mega bases in size and contains about 2000 genes (Parkhill, Achtman et al. 2000; Tettelin, Saunders et al. 2000; Bentley, Vernikos et al. 2007; Schoen, Tettelin et al. 2009). Mobile genetic elements, including IS elements and prophage sequences, make up ~10% of the genome (Parkhill, Achtman et al. 2000). The GC percentage is widely variable along the chromosome, with defined regions of low GC content that likely have been acquired by a relatively recent horizontal gene transfer. These events are common in *N. meningitidis* due to its natural transformation competence (Maiden 1993). The acquisition of the capsule locus by horizontal gene transfer, possibly from *Pasteurella multocida* or *P. hemolytica* (Schoen, Blom et al. 2008), appears to be a major event in the evolution of the pathogenicity of the meningococcus (Stephens 2009). Other than the genes encoding for the capsule, no core pathogenome has been identified suggesting that virulence may be dependent on multiple redundant genes. The most evident characteristic of neisserial genome is the high abundance of repetitive DNA sequences which leads to genetic instability, facilitating duplication or deletion of regions in the genome, as well as recombination (Davidsen and Tonjum 2006). These events characterize the non-clonal behaviour of *N. meningitidis.*

1.4 Colonization and Invasion

The first step in meningococcal colonization is the initial contact with nasopharyngeal epithelial cells mediated by Type IV pili, then bacteria proceed to proliferate on the surface of human non-ciliated epithelial cells, forming small microcolonies at the site of initial attachment (Stephens 2009). After the initial colonization, there is a loss or down regulation of the capsule, which sterically masks the outer membrane proteins. This event can occur both via regulatory system upon cell contact (Deghmane, Giorgini et al. 2002), and by selection of low or no-capsule expressing bacteria due to phase variation (Hammerschmidt, Muller et al. 1996). Close adherence of meningococci to the host epithelial cells is mediated by a variety of possible redundant adhesins, previously masked by the capsule. This results in the appearance of cortical plaques and the recruitment of factors leading to the formation and extension of epithelial cell pseudopodia that internalize the bacteria (Stephens 2009). This intracellular lifestyle can give the bacteria the opportunity to evade host immune response, find more available nutrients and is also a way to further cross the epithelium and enter the blood stream (Stephens 2009). In this new environment meningococcus has to express again the capsule, which can prevent antibody and complement deposition (Achtman 1995), is anti-opsonic and anti-phagocytic and therefore aids survival in blood (Virji 2009). These later steps in invasion of the blood stream and the possible subsequent crossing of the blood-brain barrier are still poorly understood.



(Virji 2009)

Figure 2: Stages in the pathogenesis of *N. meningitidis*. *N. meningitidis* may be acquired through the inhalation of respiratory droplets. The organism establishes intimate contact with non-ciliated mucosal epithelial cells of the upper respiratory tract, where it may enter the cells briefly before migrating back to the apical surfaces of the cells for transmission to a new host. Asymptomatic carriage is common in healthy adults in which bacteria that enter the body by crossing the epithelial barrier are eliminated. Besides transcytosis, *N. meningitidis* can cross the epithelium either directly following damage to the monolayer integrity or through phagocytes in a 'Trojan horse' manner. In susceptible individuals, once inside the blood, *N. meningitidis* may survive, multiply rapidly and disseminate throughout the body and the brain. Meningococcal passage across the brain vascular endothelium (or the epithelium of the choroid plexus) may then occur, resulting in infection of the meninges and the cerebrospinal fluid (Nassif 1999).

1.5 Virulence factors

The only known virulence factors that characterize pathogenic strains are mainly two: the polysaccharide capsule, which is thought to protect meningococci during airborne transmission between hosts (Virji 2009), allow survival in the blood as mentioned before and may shield bacterial surface from the host immune effectors mechanisms. The second principal virulence factors are the long surface proteins that protrude from the capsule known as pili. These facilitate first adhesion to host tissues apart from being the mediators of DNA uptake (Virji 2009). Pilin of Neisseria is expressed from the *pilE* locus, but homologous recombination between the *pilE* gene and a number of non-expressed 'silent' *pilS* genes results in a change in the *pilE* sequence. These variants differ in their transformability, adherence and immunogenicity (Virji, Alexandrescu et al. 1992). Additionally, *Neisseria* spp. possess host specific iron acquisition mechanisms and numerous immune evasion mechanisms such as factor H binding protein that is able to downregulate complement deposition (Virji 2009). Close adhesion and invasion is mainly mediated by an array of proteins such as opacity proteins (Opc, Opa) and other adhesins. These proteins are believed to be responsible for the host specificity as well as for tissues within the host (Virji 2009). Host specificity poses a problem for developing animal models of the disease, and as a result most of our knowledge of the pathogenic mechanisms of *Neisseria* spp. comes from *in vitro* investigations. Numerous additional apparently minor adhesins (several of which were identified by homology searching of the available genomes) are generally expressed at low levels during *in vitro* growth but may be important in *in-vivo* infections. For example, in restricted iron environments, such as might be encountered *in vivo*, the transcriptome of *N. meningitidis* is considerably altered (Grifantini, Sebastian et al. 2003) and as a result the minor adhesins may become 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 (Virji 2009). This requires a multiplicity of adhesins (redundancy) to maintain colonization (Martin, van de Ven et al. 2003; Martin, Sun et al. 2004). Several adhesins may also operate simultaneously to increase the avidity of bacterial binding to the cell surface. This is often a prelude to internalization into epithelial cells (Griffiths, Bradley et al. 2007), which can be another immune evasion strategy.

1.6 Phase Variation

Phase variation is a process that results in differential expression of one or more genes and results in two subpopulations within a clonal population: one lacking or having a decreased level of expression of the phase variable gene(s) and the other subpopulation expressing the gene fully. In this way the bacterial population can alter its antigenic properties. This process is distinct from antigenic variation, however, in which a bacterial structure, proteinaceous or otherwise, is consistently produced, but in different antigenic forms. In specific cases, phase variation can lead to antigenic variation, for example if phase variation affects expression of a lipopolysaccharide (LPS) modifying enzyme such as SiaD (Hammerschmidt, Hilse et al. 1996). A key feature of phase variation is that the 'On' and 'Off' phenotypes are interchangeable. Thus, a cell with gene expression in the 'Off' phase, that is lacking expression, retains its ability to switch to 'On' and *vice versa*. The frequency with which the switching occurs can vary widely in different systems, ranging from as frequently as one cell in ten per generation to as infrequent as one in ten thousand. The occurrence of phase variation thus results in a heterogenic and dynamically changing phenotype of a bacterial population. A change in the ratio of the two subpopulations could in principle be achieved either through

selection for or against a subpopulation, or through regulation of gene expression or of the switch frequency (van der Woude 2006).

Phase variable genes in *N. meningitidis* are typically associated with the presence of repetitive DNA motifs (oligonucleotide repeats and microsatellites) that exhibit high mutation rates by a slipped-strand mispairing (SSM) mechanism. The presence of repeat units cause a slippage of the synthesis strand over the template strand during replication that leads to the addition or the deletion of units in the new born filament (Figure 3) (Davidsen and Tonjum 2006). When repeats occur in the coding sequence, the promoter region or close to the promoter region, they can change the transcriptional and translational state of the gene, leading to an on/off switching of the gene product. In *N. meningitidis* a considerably high quantity of phase variable genes exist, affecting all kind of virulence factors or host-pathogen interacting structure such as capsule biosynthesis (Hammerschmidt, Muller et al. 1996), pili and pilus modification (Rytkonen, Albiger et al. 2004), several surface proteins including Opa, Opc, PorA and iron binding proteins (van der Ende, Hopman et al. 1995) (Lewis, Gipson et al. 1999) (Martin, van de Ven et al. 2003) (Virji 2009). It has been proposed that in *N. meningitidis* over 100 genes are potentially phase variable (Snyder, Butcher et al. 2001) (Martin, van de Ven et al. 2003), whereas for example *H. influenzae* or *E. coli* contains less than 20 and 10 putative phase variable genes, respectively (Hood, Deadman et al. 1996) (Blatter et al. 1997).

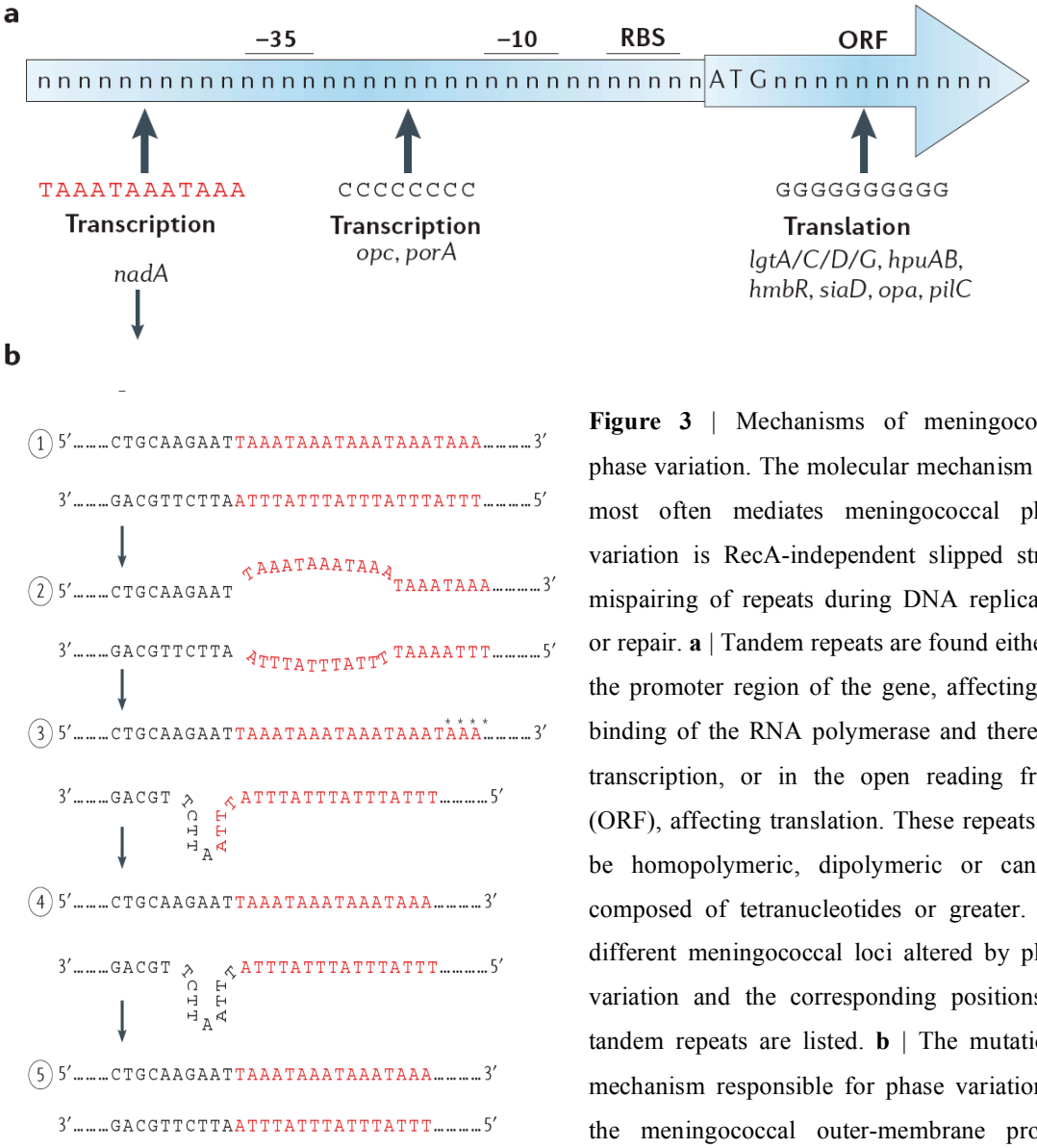


Figure 3 | Mechanisms of meningococcal phase variation. The molecular mechanism that most often mediates meningococcal phase variation is RecA-independent slipped strand mispairing of repeats during DNA replication or repair. **a** | Tandem repeats are found either in the promoter region of the gene, affecting the binding of the RNA polymerase and therefore transcription, or in the open reading frame (ORF), affecting translation. These repeats can be homopolymeric, dipolymeric or can be composed of tetranucleotides or greater. The different meningococcal loci altered by phase variation and the corresponding positions of tandem repeats are listed. **b** | The mutational mechanism responsible for phase variation of the meningococcal outer-membrane protein and adhesin NadA is shown.

(1) When 5 TAAA repeats are present upstream of the promoter region, the expression level of NadA is high (Feil, Holmes et al. 2001). (2) The AT-rich sequence is prone to strand separation. (3) Subsequent mispairing of the TAAA repeats can occur during re-annealing due to slippage of the DNA strands. Slippage in the 5′ to 3′ direction as shown leaves an unpaired copy of TAAA (indicated by the asterisks). (4) The unpaired TAAA is deleted, and the single-stranded loop is the target of excision-repair processes. (5) With a reduction in the TAAA repeat number to four, the expression level of NadA is low (Feil, Holmes et al. 2001) (Davidsen and Tonjum 2006).

1.7 Vaccines available

Effective capsular polysaccharide-based vaccines are today available for four of the five disease-associated serogroups. For serogroup B that is predominant in many countries, especially Europe and North America, no broad effective vaccine is currently available. This is due to the identity of the serogroup B capsular polysaccharide with a widely distributed human carbohydrate ([2→8]N-acetyl neuraminic acid or polysialic acid), which is a self antigen, and therefore, is poorly immunogenic in humans. Furthermore, the use of this polysaccharide in a vaccine may elicit autoantibodies (Hayrinen, Jennings et al. 1995) (Finne, Bitter-Suermann et al. 1987).

To overcome these issues, an *in-silico* genome-based approach, called Reverse Vaccinology has been used, allowing the identification of approximately 600 potentially surface exposed antigens in *N. meningitidis* serogroup B (Pizza, Scarlato et al. 2000). Among these, candidates that could be expressed in *E. coli*, shown to be surface exposed, capable of inducing bactericidal antibodies in mice, conserved and with low heterogeneity in a wide range of meningococcal strains, have been selected for further studies (Comanducci, Bambini et al. 2002) (Masignani, Balducci et al. 2003; Welsch, Moe et al. 2003). This led to the development of the 5-component vaccine against MenB, 5CVMB, which combines five antigens: GNA 2132 (Welsch, Moe et al. 2003), factor H-binding protein (FHBP) (Masignani, Comanducci et al. 2003) (Fletcher, Bernfield et al. 2004) (Madico, Welsch et al. 2006), the invasin NadA (Capecchi, Adu-Bobie et al. 2005) (Comanducci, Bambini et al. 2002), GNA 1030 and GNA 2091 (Rinaudo, Telford et al. 2009).

Study of regulation of expression of these genes could allow us not only in the better understanding of the pathogenesis of this bacterium, but has also fundamental

implications in the Meningococcal B vaccine coverage. Among the five antigens that compose the MenB vaccine, this thesis focuses on the regulation of NadA expression.

1.8 NadA

Bioinformatic analysis of the genome of a virulent *N. meningitidis B* strain (Tettelin, Saunders et al. 2000) allowed the identification of previously unknown surface proteins (Pizza, Scarlato et al. 2000), among which is the 45-kDa *N. meningitidis Adhesin A* (NadA). Structure prediction and homology comparison suggests that NadA belongs to the group of oligomeric coiled-coil adhesins (OCA) such as YadA of *Yersinia enterocolitica* and UspA2 of *Moraxella catarrhalis* (Comanducci, Bambini et al. 2002). Within these homotrimeric outer-membrane proteins, three structural regions are present: a conserved –COOH terminal membrane anchor, having a β structure; an intermediate coiled-coil stalk comprising a leucine zipper; and a –NH2 terminal region, forming the binding site(s) for target cell receptors (Hoiczyk, Roggenkamp et al. 2000). NadA is a risk factor for the development of meningococcal disease, as it was found in 50% of *N. meningitidis* strains isolated from patients and in only 5% of strains from healthy individuals (Comanducci, Bambini et al. 2004). NadA has been implicated in the mucosal colonization by *N. meningitidis*, as its expression enhances bacterial adhesion to and invasion of mucosal cells (Capecchi, Adu-Bobie et al. 2005). NadA has also been implicated in interaction and stimulation of monocyte, macrophage and dendritic cells during *N. meningitidis* infection (Franzoso, Mazzon et al. 2008).

Little is known about the factors or mechanisms regulating expression of the NadA protein. Its expression was shown to exhibit growth-phase dependent behaviour with levels reported to be maximal in the stationary growth phase of all strains tested (Comanducci, Bambini et al. 2002). Furthermore, the expression of NadA is phase

variable and a tetranucleotide tract (TAAA) upstream of the *nadA* gene promoter has been demonstrated to control this phenomenon (Martin, van de Ven et al. 2003).

The phase variable tract of *nadA* is distally located upstream of the *nadA* promoter, unlike the phase variable repeat tracts found in the *porA, fetA*, and *opc* genes where the unstable homopolymeric stretches are found between the -10 and the -35 promoter elements and are thought to result in altered sigma-factor binding (Sarkari, Pandit et al. 1994) (van der Ende, Hopman et al. 1995) (Carson, Stone et al. 2000). The phase variation mechanism for the *nadA* gene is unique in that all phase variable promoter variants result in the expression of full length protein, which is unlike the case when repeats are located in coding regions (frame-shift mutation leading to generation of stop codons) or when suboptimal -10 and -35 promoter spacing precludes or affects promoter recognition or activation. The frequency of phase variation of *nadA* has been experimentally estimated as ca. 4.4×10^{-4} (Martin, van de Ven et al. 2003), creating variants where changes in the repeat number result in promoters with low, medium or high activity. The transcriptional regulators Fur and IHF were implicated in the control of *nadA* promoter activity from the specific binding of both proteins to the *nadA* promoter and from the analysis of mutants deleted for multiple IHF- and Fur-binding sites (Martin, Makepeace et al. 2005). Moreover, it has been reported that loss or gain of a tetranucleotide repeat affects the binding of the IHF regulatory protein to the *nadA* promoter *in vitro*, and this was proposed to be responsible for the modulation of transcription of *nadA in vivo* (Martin, Makepeace et al. 2005). Nonetheless, the precise mechanism governing transcriptional regulation of *nadA* remains unclear and the inferred role of IHF or Fur and their involvement in phase variation of *nadA* expression remain to be elucidated. However, a novel regulator of NadA expression has recently been identified which was shown to repress NadA expression (Schielke, Huebner et al. 2009).

In this work we will show results on the interactions on *nadA* promoter region, and the involvement in its transcriptional regulation, of three different regulatory factors: the α-subunit of RNA polymerase, a MarR family transcriptional regulator named NadR (NMB1843) and the architectural protein IHF.

1.9 Alpha subunit of RNA polymerase

In recent years, it has become clear that promoter recognition by bacterial RNA polymerase (RNAP) involves interactions not only between core promoter elements and the sigma subunit, but also between a DNA tract, called UP element, upstream of the −35 sequence and the RNAP α subunit (Hawley and McClure 1983) (Ross, Gosink et al. 1993) (Blatter, Ross et al. 1994). The best-characterized UP element is in the *rrnB* P1 promoter, in which the sequence determinants are located between positions −40 and −60 with respect to the transcription start site (Rao, Ross et al. 1994), and UP element-α-subunit interactions facilitate initial binding of RNAP and subsequent step(s) in transcription initiation (Rao, Ross et al. 1994) (Strainic, Sullivan et al. 1998). A consensus UP element sequence derived from binding-site selection experiments, consists almost exclusively of A and T residues and increases promoter activity >300-fold (Estrem, Gaal et al. 1998). Each RNAP α subunit consists of two domains connected by an unstructured and flexible linker (Blatter, Ross et al. 1994) (Jeon, Yamazaki et al. 1997). The 28-kD aminoterminal domain (αNTD) is responsible for dimerization of α and for interaction with the remainder of RNAP (Igarashi and Ishihama 1991; Busby and Ebright 1994). The 8-kD carboxy-terminal domain (αCTD) is responsible for interaction with the UP element (Blatter, Ross et al. 1994) and with a number of transcriptional activators (Igarashi and Ishihama 1991) (Busby and Ebright 1999) (Savery, Lloyd et al. 1998). The αCTD residues most crucial for DNA interaction are nearly invariant in bacteria (Gaal, Ross et al. 1996) (Murakami, Fujita et al. 1996),

and therefore the DNA sequences recognized by α are also likely to be highly conserved. The interdomain linker presumably accounts for the ability of αCTD to interact with DNA and/or activator molecules at different locations upstream of the −35 element (Newlands, Josaitis et al. 1992) (Blatter, Ross et al. 1994) (Murakami, Owens et al. 1997) (Belyaeva, Rhodius et al. 1998) (Hochschild and Dove 1998) (Law, Savery et al. 1999).

1.10 MarR family transcriptional regulators

The MarR (multiple antibiotic resistance regulator) family of prokaryotic transcriptional regulators includes proteins critical for control of virulence factor production, bacterial response to antibiotic and oxidative stresses and catabolism of environmental aromatic compounds. MarR proteins exist as homodimers in both free and DNA-bound states. Sequence specific DNA-binding to palindromic or pseudopalindromic sites is mediated by a conserved winged helix fold and, for numerous homologs, this association is attenuated by specific anionic lipophilic ligands. 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. Proteins belonging to this family of transcriptional regulators serve physiological roles as sensors of changing environments, a capacity particularly critical for pathogenic bacteria. Homologs of MarR are 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 (Perez-Rueda and Collado-Vides 2001) (Perez-Rueda, Collado-Vides et al. 2004). Transcriptional regulation by MarR proteins is modulated by specific anionic lipophilic (usually phenolic) compounds, which attenuate the ability of MarR homodimers to bind their cognate DNA sequences. The gene encoding each

MarR homolog is generally part of a gene cluster containing the gene(s) under its regulation (with a couple of notable exceptions) and in some cases, the MarR homolog is encoded in its regulated operon. 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 (Stapleton, Norte et al. 2002) (Galan, Kolb et al. 2003). Cooperative binding to closely spaced recognition sequences has been demonstrated for OhrR from *Bacillus subtilis* and may occur for other MarR homologs, as well (Evans, Adewoye et al. 2001) (Fuangthong and Helmann 2002) (Stapleton and Taylor 2002). The physiological roles of MarR proteins can be classified into three general categories with some proteins serving multiple regulatory roles: 1) regulation in response to environmental stress, 2) regulation of virulence factors, and 3) regulation of aromatic catabolic pathways (Wilkinson and Grove 2006). A MarR homolog has been characterized from *N. gonorrhoeae* that likely mediates the resistance of this organism to antimicrobial hydrophobic agents. FarR represses its own transcription and that of the distally located *farAB* operon of *N. gonorrhoeae*, which encodes an efflux pump that exports host-derived antimicrobial agents such as long-chain fatty acids (Lee, Rouquette-Loughlin et al. 2003).

Similarly, HpaR from *E. coli* represses transcription of the *hpa-meta* operon which encodes genes for the catabolism of 4-hydroxyphenylacetic acid and this repression is relieved by 4-hydroxyphenylacetic acid and structurally similar compounds (Galan, Kolb et al. 2003).

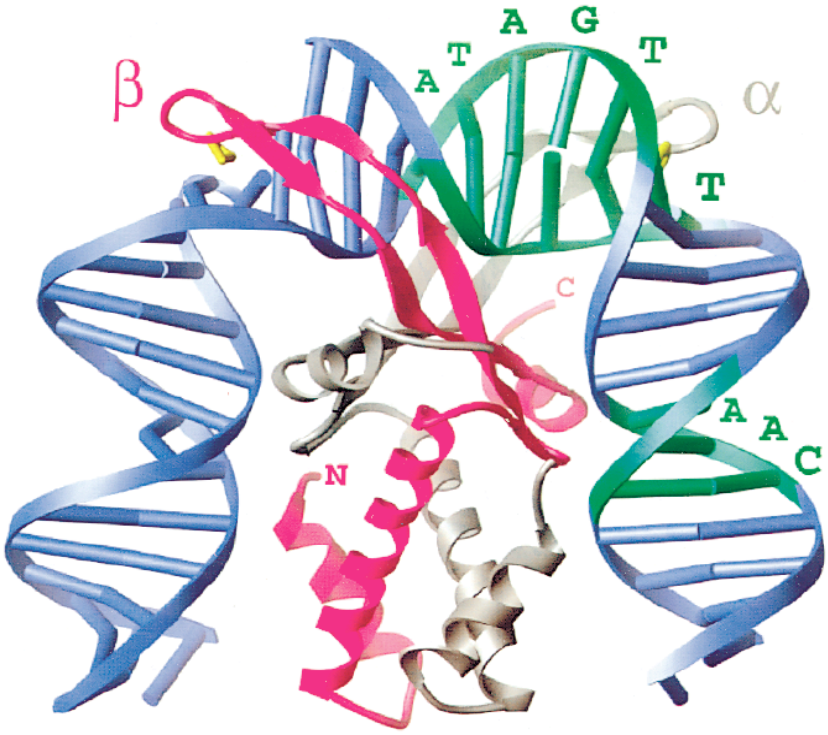
1.11 IHF

For macromolecular complexes built on a DNA template, the active components are often insufficient to generate the proper architecture, and accessory factors are needed. Integration host factor (IHF) is a small heterodimeric protein that specifically binds to DNA and functions as an architectural factor in many cellular processes in prokaryotes (Rice, Yang et al. 1996).

IHF is composed of two subunits, which share about 25% homology. The alpha-subunit is approximately 11kDa and the beta-subunit about 9.5kDa in size. The structure of the hetero-dimeric protein has been resolved and can be described as a body with protruding flexible arms that can be inserted into the minor groove of DNA (Rice, Yang et al. 1996) (Swinger and Rice 2004). A typical specific IHF binding site is approximately 30 bp. The 3' region is conserved (matching the consensus (A/T)ATCAANNNNTT(A/G) (where N=any nucleotide)), whereas the 5' region is not conserved, although commonly A/T rich (Goodrich, Schwartz et al. 1990) (Luijsterburg, Noom et al. 2006).

Although first discovered as a host factor for bacteriophage lambda integration, IHF assists in many processes that involve higher order protein–DNA complexes: e.g., in replication, where it binds to *oriC*; in transcriptional regulation, where it binds upstream of many σ54-dependent promoters; and in a variety of site specific recombination systems (Rice, Yang et al. 1996) (Goosen and van de Putte 1995). IHF's primary function appears to be architectural, i.e., introducing a sharp bend in the DNA that facilitates the interaction of other components in a nucleoprotein array (Figure 4). Where tested, IHF can be at least partially replaced by heterologous DNA-bending proteins or by intrinsically bent DNA (Goodman, Nicholson et al. 1992) (Molina-Lopez, Govantes et al. 1994) (Perez-Martin, Timmis et al. 1994) (Segall, Goodman et al. 1994) (Parekh and Hatfield 1996) (Rice, Yang et al. 1996).

Interestingly, IHF has been shown to enhance transcription levels from *pilEp1* promoter in *N. gonorrhoeae* with the possible involvement of two UP elements (Fyfe and Davies 1998). Moreover, in *Pseudomonas putida*, IHF binding mediate a topological switch that governs the positioning of RNA polymerase alpha subunit over 2 distinct UP elements in Pu promoter (Macchi, Montesissa et al. 2003). Finally it has been shown that IHF can bind over *nadA* promoter in *N. meningitidis* (Martin, Makepeace et al. 2005).



(Rice, Yang et al. 1996)

Figure 4. Complex of IHF with Site H91N. (A) Front view. The alpha subunit is shown in white; beta, pink. The consensus sequence is highlighted in green and interacts mainly with the arm of alpha and the body of beta. The yellow proline at the tip of each arm (P65a/P64b) is intercalated between bp 28 and 29 on the left side and 37 and 38 on the right.

Results

1.12 Phase variation in expression and transcript level of NadA

Previous analysis of NadA expression in several meningococcal isolates indicated that its expression is controlled by variation in the number of tetranucleotide repeats (TAAA) upstream of the core promoter (Martin, van de Ven et al. 2003) and that the protein is maximally expressed in stationary growth phase (Comanducci, Bambini et al. 2002).

Figure 5A shows key elements of the *nadA* promoter sequence, identified in this work through footprinting analysis (shaded boxes), as well as positions of different deletions in the promoter (ΔP from 2 to 5).

First of all, to confirm this data, we analyse NadA expression level in different *N. meningitidis* strain harbouring different numbers of repeats in the *nadA* promoter. As shown in the western blot of Figure 5B, from overnight bacteria grown on plates, NadA is expressed at low level in MC58 strain (carrying 9 TAAA repeats upstream of the promoter), intermediate level in 961-5945 and ISS838 (having 12 and 6 repeats, respectively) and high level in strains BZ83 and 5/99 (5 and 8 repeats, respectively).

In order to rule out any possible effect in NadA expression due to strain differences we decided to study transcriptional regulation of the *nadA* promoter generating isogenic *N. meningitidis* MC58 strains, carrying a complete range of *nadA* phase variant promoter fusions with a different number of repeats and determined the relative level of the transcripts from these promoters. Steady state levels of *nadA* transcript were measured by quantitative primer extension analyses in cells grown to the mid log and the stationary growth phases.

As is evident from Figure 5C, the transcript level from the *nadA* promoter gives a quasi periodic pattern with 4, 9 and 12 repeats resulting in the lowest transcription, 7, 8 and 10 has instead the highest transcript level, and 5, 6, 11, 13 and a promoter mutant lacking TAAA repeats (pΔ) giving varying intermediate levels. These results mirror the NadA expression levels seen in the five strains of panel A, strongly suggesting that different expression levels could be due only to differences in TAAA repeats number.

Furthermore, each phase variant promoter exhibits a certain degree of growth-phase dependent transcription, with a maximum level of transcription in stationary growth phase from promoters harbouring 7, 8 and 10 repeats.

Figure 5

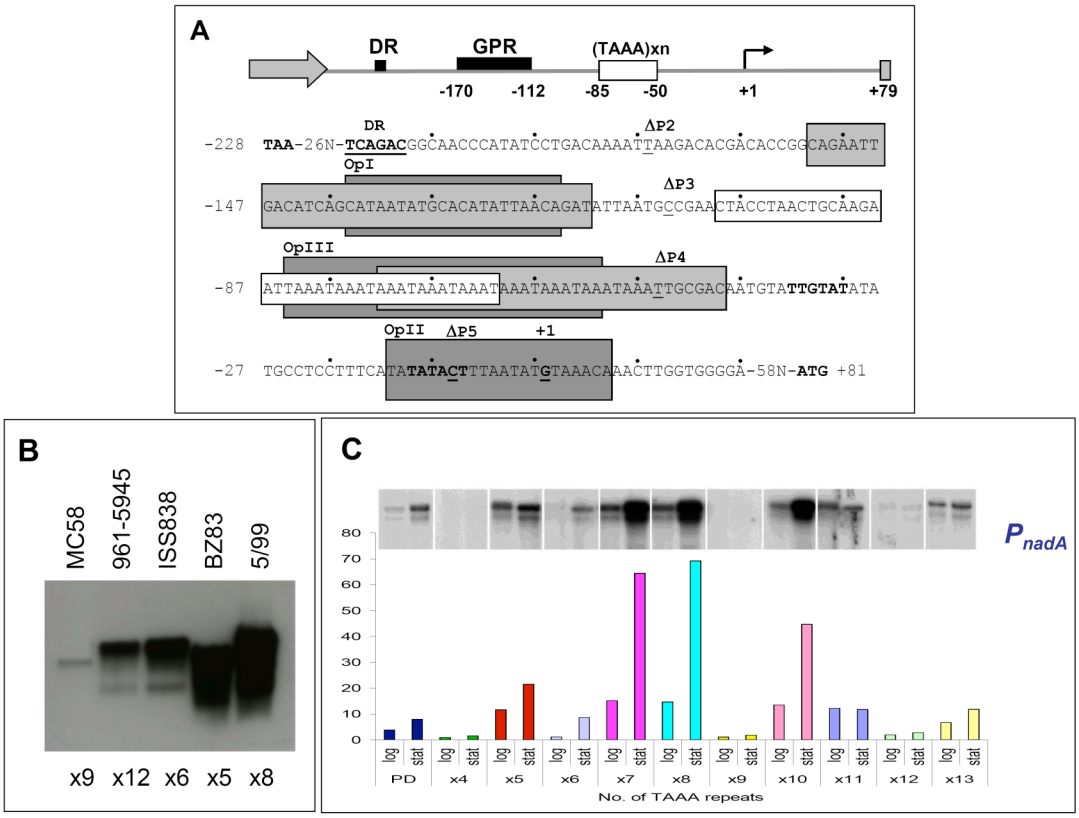


Figure 5: The P_{nadA} promoter and transcript level. A) Schematic diagram of the P_{nadA} elements. DR, direct repeat (border of region of horizontal transfer); GPR, growth phase regulatory region; ΔP2-ΔP5 indicates the nucleotide positions of the 5' deletion mutants (Figure 8C). The nucleotide sequence of the promoter is shown with the regions bound and protected in DNase I footprinting experiments shaded according to the regulatory proteins tested in vitro: light grey, RNAP α-subunit; white, IHF; dark grey, NadR. (B) Western Blot analysis of the level of expression of NadA in wild type strains 5/99, BZ83, ISS838, 961-5945 and MC58 carrying *nadA* promoters with 8, 5, 6, 12, and 9 repeats, respectively. Cells were recovered from overnight culture on plates and 5 μg of total protein were loaded on SDS-PAGE, blotted and stained with anti-NadA polyclonal antiserum. (C) Transcription of each phase variant promoter is growth phase responsive. Cultures of MC-PΔ, MC-P2(x4), MC-P2(x5), MC-P2(x6), MC-P2(x7), MC-P2(x8), MC-P2(x9), MC-P2(x10), MC-P2(x11), MC-P2(x12), MC-P2(x13) strains, carrying single copy transcriptional fusions of the phase variant *nadA* promoter with a defined number of copies of the tetranucleotide repeat (TAAAxN) and the repeated tract deleted (Δ) (Table II), were grown to mid-log or stationary growth phase and total RNA was prepared. Quantitative primer extension was performed as described in materials and methods. Autoradiographs of a representative experiment are shown as well as the quantification of transcript levels as determined by phosphorimaging.

1.13 RNA polymerase interaction over the *nadA* promoter

The repeated tract in the *nadA* promoter, due to its high content in AT and its localization upstream the -35 sequence, can be a perfect candidate as an UP-element, thus influencing transcription through interaction with the α−subunit of the RNA polymerase (RNAP). In order to gain insight into the interaction of the polymerase with *nadA* promoter region we performed footprinting experiments using the holoenzyme and three radioactively labelled phase variant promoters, corresponding to low (9 repeats), medium (6 repeats), and high (7 repeats) transcript level.

As expected, addition of RNAP to the *nadA* promoter probe resulted in a characteristic footprint over the core promoter spanning from -37 to +17, as well as protecting two other regions, one directly upstream of the core promoter spanning positions -43 to -76, partially overlapping the TAAA tract, and the second distally upstream spanning from -116 to -154 with respect to the probe with 9 repeats (Figure 6A). Notably, at least three hypersensitive bands appear between the two protected regions upstream of the promoter, suggesting that there is a distortion in the DNA of this region upon binding of the RNAP. Interestingly, no relevant differences in the protected regions are visible between the phase variant promoters tested, with the exception of the PΔ promoter variant, deleted of the TAAA tract, in which the region just upstream the core promoter is not protected anymore (Figure 6B).

As both upstream protected areas are AT-rich regions, a typical feature of UP-like elements bound by the C-terminal region of the α-subunit of RNAP to enhance transcription (Ross, Aiyar et al. 1998) (Estrem, Gaal et al. 1998), we decided to verify such a hypothesis in vitro by DNase I footprinting using the purified α-subunit of the RNAP.

Results showed a specific binding of the α protein over the TAAA repeats at low protein concentration (Figure 6A). Upon addition of increasing amounts of the α protein, this protected area extended both to downstream and upstream regions, including regions spanning positions -43 to -76 and -116 to -154 protected by the holoenzyme (Figure 6A).

Furthermore, because the *nadA* promoter is recognised and transcribed from the same +1 in *E. coli* (data not shown), we decided to test whether the α-subunit of RNAP could play a role in the transcription of P*nadA* in this system. We measured promoter activity of a P*nadA-gfp* fusion (on plasmid pGX-nad- gfp) in an *E. coli* strain over-expressing either a wild type α-subunit (RpoA) or a C-terminally truncated α–subunit (RpoAΔ256) of *E. coli*. Expression of the P*nadA-gfp* fusion in the strain over-expressing the wild type α-subunit gave 6393 ± 254 Units (fluorescence normalized with OD600), while in the strain over-expressing the α truncated version, the activity was reduced by over 50% giving 2867 ± 63 Units. No reduction in promoter activity was apparent when the PΔ *nadA* fusion was co-expressed with the α or truncated α subunit (Figure 6C).

These data suggest that the incorporation of a complete α-subunit into the RNAP allows maximum transcriptional activity at P_{nadA}, possibly through contacts of the C-terminal region of the α-subunit to upstream DNA regions containing AT-rich sequences sharing similarities to an UP element. However, the roles of the distal region of interaction with the α-subunit, as well as possible effects of different DNA topology removing the TAAA tract, remain to be elucidated.

Figure 6

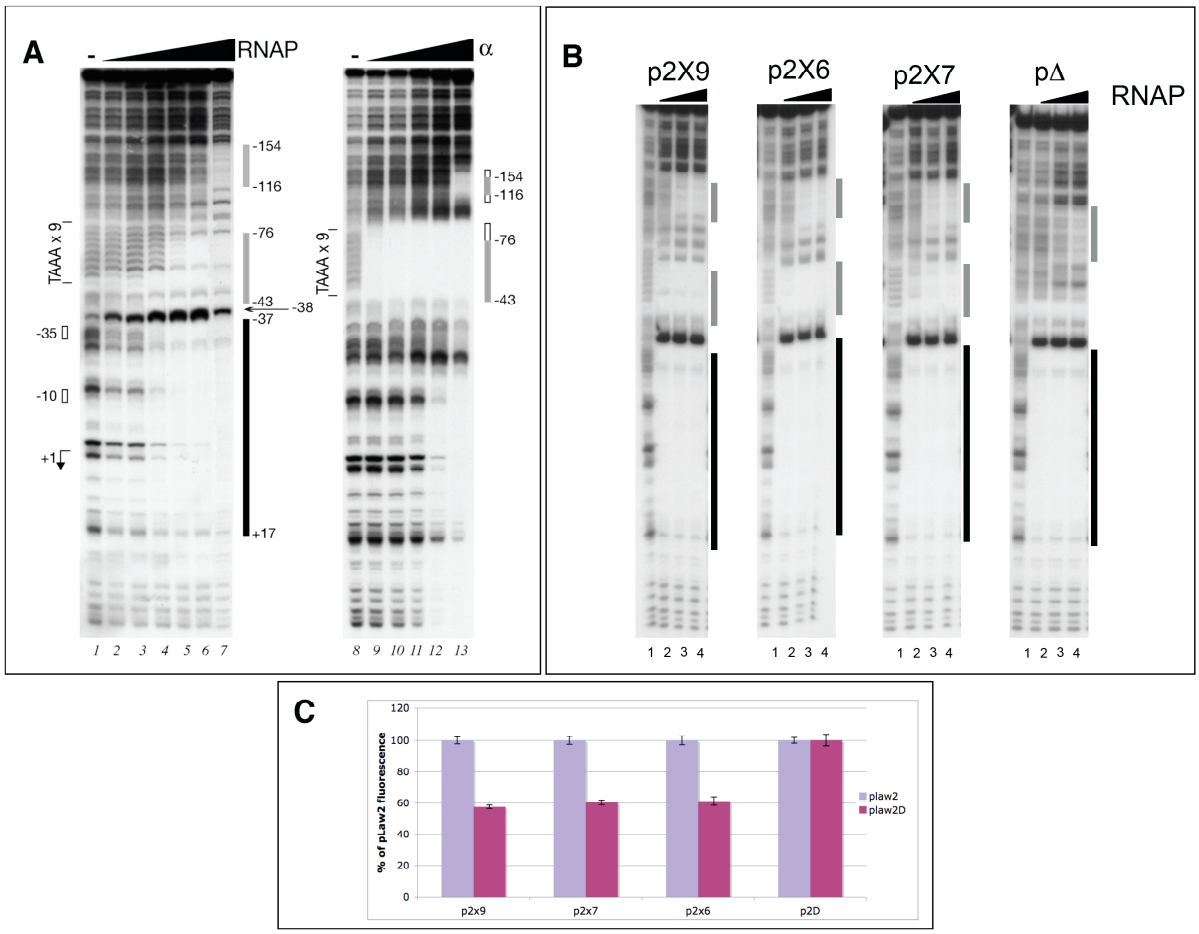


Figure 6: RNA polymerase interaction over NadA promoter. (A) DNase I footprinting of RNAP or the α-subunit of RNAP to the indicated *nadA* promoter probe. The probe was incubated with 0, 0.25, 0.5, 1, 2, 4, and 5 U of RNAP (lanes 1-7) or 0, 0.17, 0.68, 2.7, 5.5, 11 μM of purified α-subunit (lanes 8-13). (B) DNase I footprinting of RNAP to four different *nadA* phase variant promoters, the probes were incubated with 0, 2, 4 and 6 U of RNAP (lanes 1-4). Black bars indicate RNAP core enzyme protection, whereas grey bars indicate α-subunit of RNAP protection. (C) Fluorescence measurements of *E. coli* expressing PnadA-GFP fusions and over-expressing either wild type α-subunit or a C-terminally truncated α-subunit (plaw2 and plaw2D respectively). Bacteria were grown at mid log growth phase and then fluorescence was measured normalized with OD_{600}. Numbers are given as percentage of fluorescence with respect to strains over-expressing wild type α-subunit.

1.14 IHF interacts with the *nadA* promoter

In vitro DNA binding assays suggested that regulation of *nadA* expression is under the control of the Fur and IHF regulatory proteins and that loss or gain of TAAA repeats could affect IHF binding, thus accounting for the different promoter activity of the phase variants (Martin, Makepeace et al. 2005) Moreover, IHF seems to be involved in allowing interaction of distal UP-elements with the RNAP in *pilEp1* promoter of *N. gonorrhoeae* and in *pu* promoter of *P. putida* (Fyfe and Davies 1998) (Bertoni, Fujita et al. 1998) respectively. An involvement of IHF in *nadA* regulation, could also explain how a DNA region distally upstream (from -116 to -154) can interact with the α-subunit of the RNAP bound to the core promoter.

We mapped the precise location where Fur and IHF bind to the *nadA* promoter. DNase I footprinting was performed with the purified proteins and the same phase variant promoters used for RNAP experiments shown in Figure 6.

Addition of increasing amounts of a recombinant Fur protein (0.013-3.2 μM) showed a region of protection at 3.2 μM Fur concentration (data not shown). This protection overlaps the translational start site (+79) spanning from +61 to +96. Moreover, no differences in *nadA* transcription were detected in a Fur null mutant background when compared with the wild type strain, nor in response to changing iron concentrations (data not shown). Therefore, the observed in vitro binding of Fur to the *nadA* promoter appears to have no correlation with *in vivo* regulation of transcription by Fur in response to iron.

On addition of 43 or 172 nM of the IHF heterodimer to the binding reactions resulted in a similar region of protection in all three phase variant probes (Figure 7). IHF binds

upstream of the distal border of the TAAA tract and the protection spans the first 5 repeats, from -103 to -65 with respect to the promoter with 9 repeats (Figure 7). Accordingly, no binding could be detected in PΔ promoter variant in which the TAAA tract was deleted (Figure 7). Notably, variations of the number of repeats resulted in no differential binding of IHF in these experimental conditions.

IHF is well known for its ability to bend DNA by up to 180° (Rice, Yang et al. 1996) and this property may permit looping of the DNA and the interaction of regulators at distal operators and the transcriptional machinery over the promoter.

Figure 7

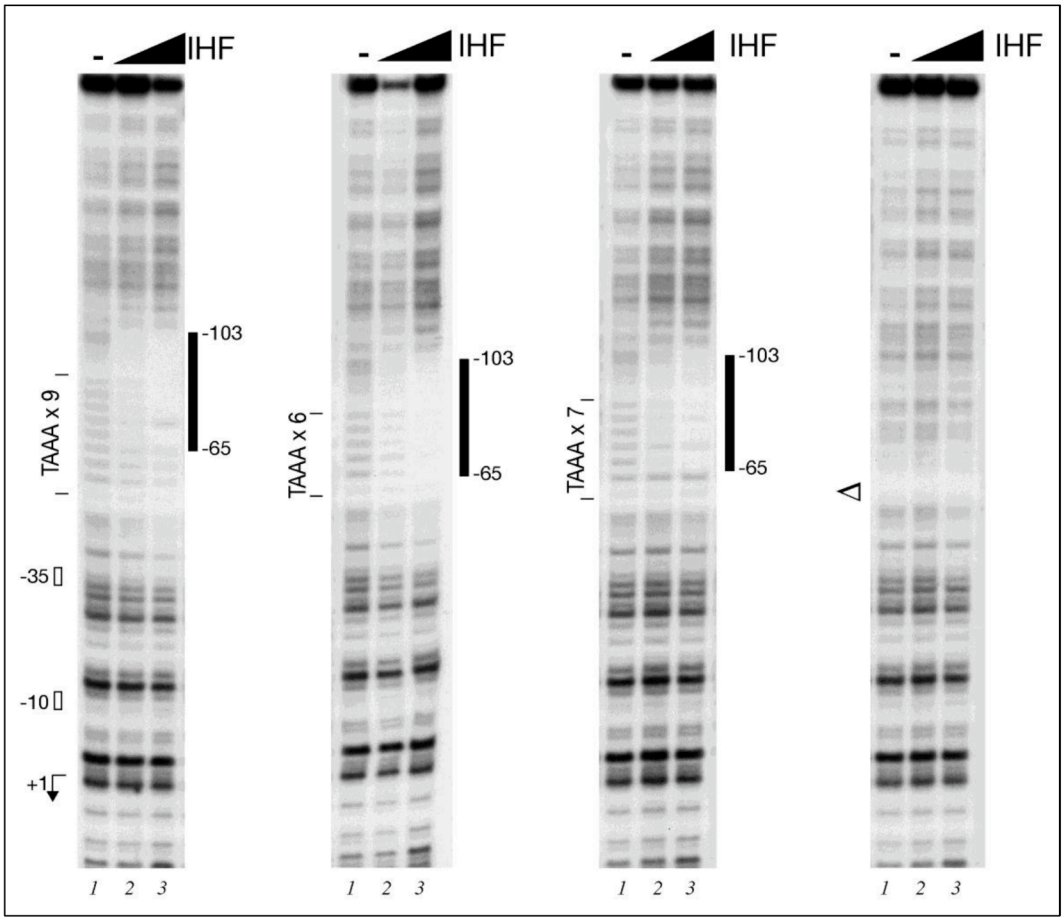


Figure 7: IHF interaction over NadA promoter. DNase I footprinting of IHF protein to three different phase variant *nadA* promoters with 9, 6 and 7 repeats corresponding to low, medium and high transcript level *in vivo*, respectively, and the PΔ mutant *PnadA* variant with a deletion of the TAAA repeated tract. To 20 fmoles of each radioactively labelled probe, 0, 43 and 172 nM (lanes 1-3) of IHF heterodimer were added. Relevant regions are marked and numbers correspond to nucleotide positions with respect to the transcriptional start site of a promoter with 9 repeats.

1.15 Identification of a *cis* acting element responsible for growth phase regulation

Transcription from the *nadA* promoter is regulated during growth, as shown in the primer extension experiment shown in Figure 8B, which was performed on total RNA extracted at different time points from MC58 growing cells. As shown, *nadA* is transcribed maximally in late logarithmic and stationary phase. Unlike adenylate kinase gene that, as many other housekeeping genes, is less transcribed entering in stationary phase due to a general slowdown in the bacterial metabolism.

In order to identify regulatory regions within the P_{nadA} promoter we created a range of deletion mutants in *N. meningitidis* MC58 strain, and measured the transcript level from cells grown to the mid-log and stationary growth phases (Figure 8C and 8D). While deletion of nucleotide sequences upstream of -170 with respect to the +1 transcriptional start site had little or no effect on the level of transcript (promoter P2 versus P1), promoter mutants lacking the region between -170 and -108, (P3 or P4) resulted in a significant increase in transcription during log phase. The same results have been obtained for promoters harbouring 11 repeats (data not shown). This finding suggests that the growth-phase dependent regulation is due to a repression of expression in log phase, more than an induction in stationary phase. Accordingly, removal of the TAAA tract did not alter the growth-phase regulation of the resultant mutants (P4 versus P3, or PΔ versus P2). Therefore, we have identified a distal upstream *cis*-acting region that we call the GPR region (for growth phase regulatory), which is responsible for repression of transcription from P_{nadA} in log phase, possibly upon binding of a repressor protein.

Figure 8

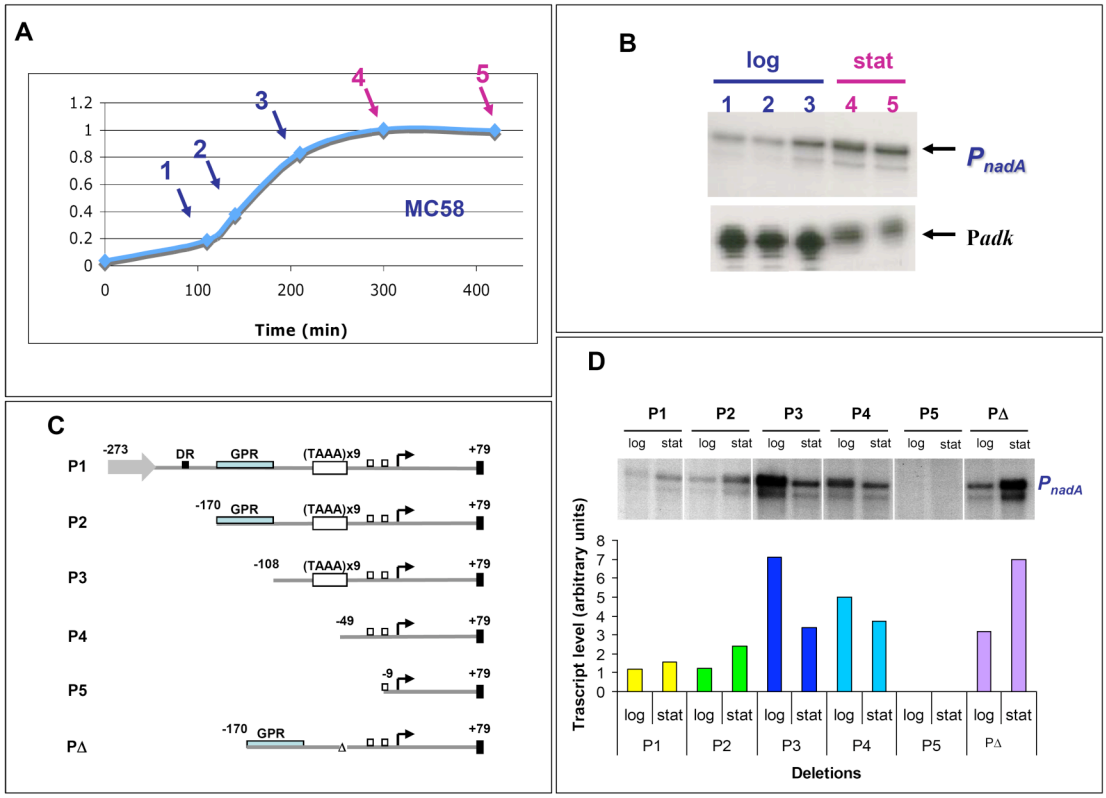


Figure 8: Identification of a *cis*-acting element of the *nadA* promoter determining growth phase regulatory effects (the GPR region). (A) Growth curve of MC58 strain. Bacteria from an overnight plate were inoculated at an OD_{600} of 0.05 in GC liquid media and grown for 7 hours. Numbers indicate the points in which RNA samples were taken. (B) Primer extension analysis on *nadA* and *adk* transcript from total RNA extracted at time-points indicated in panel A. (C) Schematic representation of the mutant *nadA* promoter variants (based on the MC58 *nadA* promoter with 9 repeats) present in single copy transcriptional fusion in the MC58 background in the strains, MC-P1, MC-P2, MC-P3, MC-P4, MC-P5, MC-PΔ. The numbers indicate nucleotide positions with respect to the +1 transcriptional start site. DR, direct repeat; GPR, growth-phase regulatory region; Δ, deletion of the TAAA repeats. (D) Transcription from the mutant promoter variant fusions in log and stationary phases. The MC-P1, MC-P2, MC-P3, MC-P4, MC-P5, MC-PΔ strains were grown to mid-log and mid-stationary growth phase and total RNA was prepared from each sample. Quantitative primer extension was performed as described in materials and methods. Autoradiographs of a representative experiment are shown as well as the quantification of transcript levels. Similar results were found for deletion variants carrying 11 TAAA repeats (data not shown).

1.16 A protein in meningococcus cell extract binds GPR region

To assess whether a repressor factor could bind the GPR region we analysed crude cell extracts of the MC58 strain for the ability to retard a radioactively labelled GPR probe in Electrophoretic Mobility Shift Assays (EMSA). Addition of 15 µg of MC58 extracts resulted in a complete shift of the GPR probe, which could be outcompeted with cold GPR DNA but not with non-specific competitor (Figure 9A). We also found that the P5 promoter probe spanning from -9 to +79 of the P_{nadA} promoter was specifically retarded (lane 11, Figure 9A) by MC58 extracts but not an unrelated intergenic region (Pcon) used as negative control.

We further characterize the nature of this binding factor performing EMSA after incubation of the cell extract with different proteases or at different temperatures. Upon treatment with 5 µg of Trypsin or ProteinaseK for 2 hours we were able to completely abolish the binding activity of the cell extract (Figure 9B, lanes 4 and 6). Furthermore, incubating the cell extract for 30 minutes at 60 or 100 °C has the same effect of protease treatment, probably due to protein denaturation, whereas 30 minutes at 40 °C does not seem to alter the binding activity. Then, we fractionate cell extract using sequential ammonium sulphate precipitation, with increasing salt concentration. The binding activity is retained only in the 70% precipitated fraction, in which there is an enrichment of small proteins. These data taken together suggest that the GPR binding factor could be a small protein relatively heat-resistant.

Figure 9

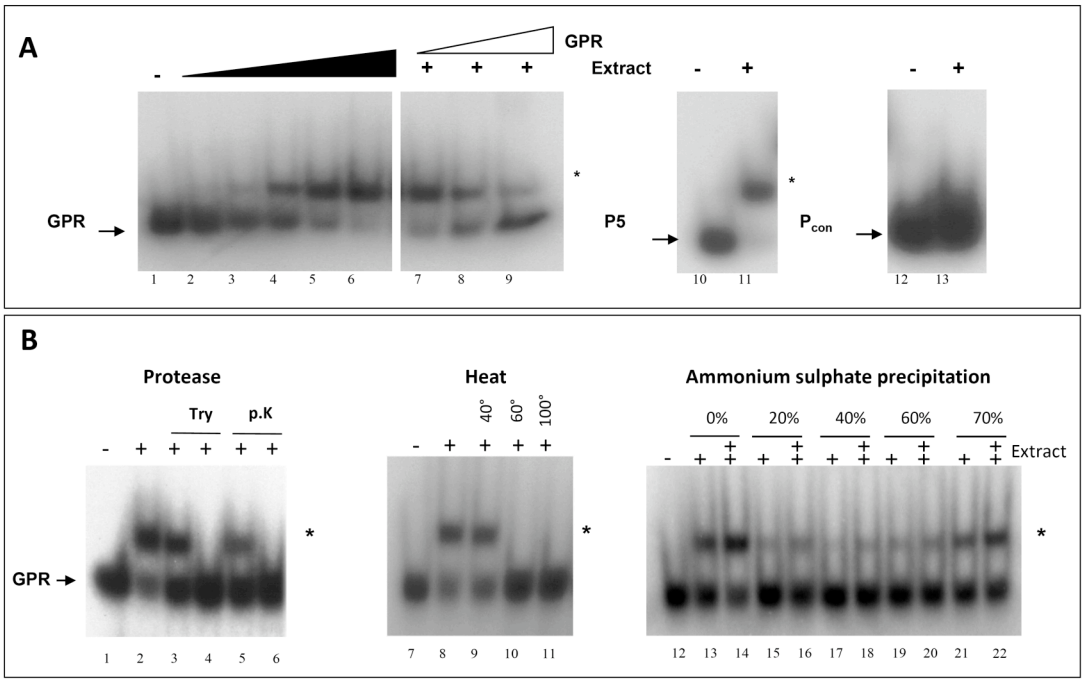


Figure 9: A protein in meningococcus cell extracts specifically binds the GPR region. (A) Binding activity towards the *nadA* promoter in cell extracts of MC58. Cell extracts were prepared from mid-log cultures of MC58 and increasing quantities were incubated with a radioactively labelled DNA probe consisting of the GPR region (-170 to -108) or P5 (- 9 to +79) or an unrelated intergenic region Pcon as negative control and submitted to EMSA analysis. To ca. 80 fmoles of radioactively labelled probe, 0, 0.2, 0.6, 1.8, 5.0, 15 μg of cell extract in lanes 1-6 were added, respectively; 0 μg in lanes 10 and 12; and 15 μg in lanes 7-9, 11 and 13, were added; and 130, 400, and 1000 fmoles of cold GPR probe in lanes 7, 8, and 9 were added as specific competitor. (B) Different treatments of the cell extract in order to characterize the nature of the binding activity. Ten μg of cell extracts were incubated with 5 μg of Trypsin or Proteinase K either for 30 minutes or 2 hours (lanes 3-6), or for 30 minutes at 40, 60 and 100 °C (lanes 9-11 respectively) and then subjected to EMSA assay. To fractionate cell extract we sequentially precipitated it with ammonium sulphate salts at 20, 40, 60 and 70% (lanes 15-22, respectively) and then perform EMSA assay using 2 μg (+) or 4 μg (++) of each fraction.

1.17 Identification of the GPR binding protein

To identify the GPR binding factor we performed DNA affinity purification using the biotinylated GPR region as 'bait' and streptavidin coated magnetic beads (Figure 10A). After incubation of the biotinylated GPR probe with the extract and the addition of streptavidin coated magnetic beads, the sample was washed and the GPR binding factor eluted with high salt. Each step of the purification was then analyzed for GPR binding activity through EMSA. Figure 10B shows as the binding activity is successfully eluted from the beads only in presence of biotinylated GPR DNA and not in the negative control (same purification performed with a non biotinylated GPR DNA). The eluted fraction was digested with trypsin, and the resulting peptides were analyzed by MALDI-TOF mass spectrometry. Four of the seven major ions could be assigned to tryptic peptides derived from the NMB1843 protein (Figure 10C and 10D). The other three peaks could be assigned to bovine serum albumin which was added in the purification step to block non-specific interaction. To confirm the interpretation, the major parental ions were fragmented. Spectra of fragmentation were consistent with the expected NMB1843 amino acid sequence (data not shown). We call this protein that binds the GPR region of the *nadA* promoter NadR. The *nadR* gene encodes a transcriptional regulator of the MarR family of repressors, is a homologue of FarR, the repressor of the fatty acid resistance efflux pump of *N. gonorrhoeae* (Lee and Shafer 1999) (Lee, Rouquette-Loughlin et al. 2003) and was recently implicated as a repressor of *nadA* (Schielke, Huebner et al. 2009). We rename the meningococcal homologue NadR as, unlike the FarR protein, it does not regulate the fatty acid efflux pump in meningococcus (Pigozzi E, personal communication) and, therefore, is not involved in fatty acid resistance.

Figure 10

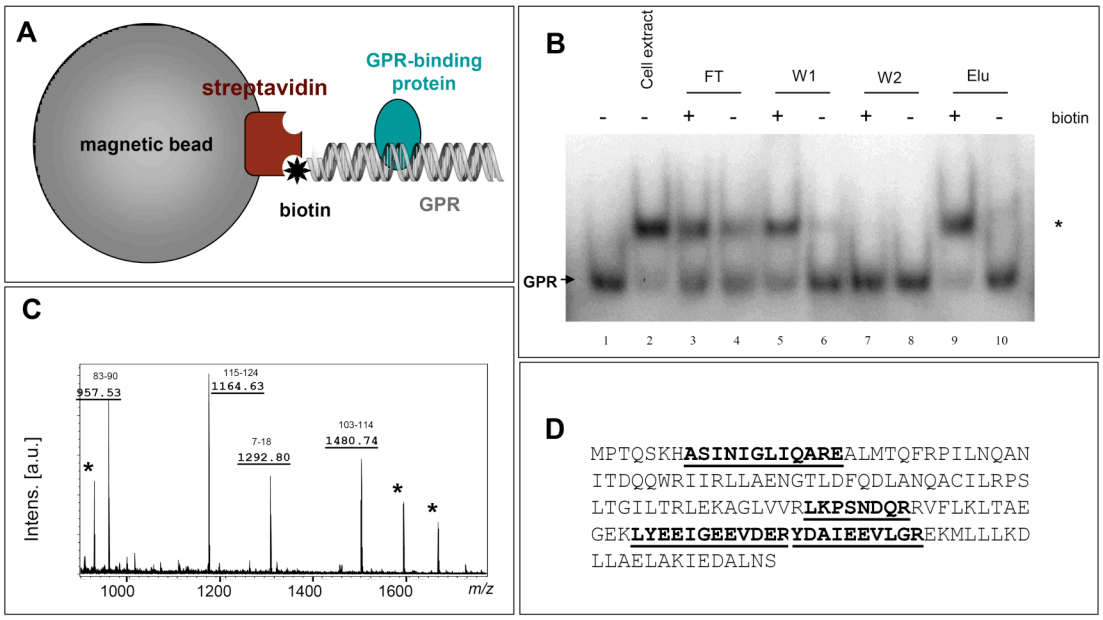


Figure 10: Identification of the GPR binding protein. (A) Cartoon showing the DNA affinity purification system. (B) To monitor the purification efficiency, every fraction was subjected to EMSA assay using an unbiotinylated GPR probe as negative control (lanes 4, 6, 8 and 10). FT: flow trough, W1 and W2: washes, Elu: eluted fraction. (C) The peptide mass fingerprint spectrum of one µl of the eluted fraction after DNA affinity purification of the binding factor of the GPR region. Four of the major ions, labelled, could be assigned to tryptic peptides (positioning of the amino acids indicated above) of the NadR transcriptional regulator protein. In addition, BSA, that was added during the process of purification, was eluted from the column, since 3 major signals observed in the spectrum corresponded to BSA tryptic peptides (marked with an asterisk). (D) The amino acid sequence of the NMB1843 (NadR) protein showing the peptides that were identified by MS in bold and underlined.

1.18 NMB 1843 interacts with the *nadA* promoter

We amplified and cloned the *nadR* gene (NMB1843) from the MC58 genome into an expression plasmid and expressed and purified a recombinant form of the protein with an N-terminal Histidine tag. We performed DNase I footprinting analysis with the NadR protein and a radioactively labelled probe consisting of the entire *nadA* promoter. Figure 11A shows the autoradiogram of the results. On addition of increasing amounts of NadR recombinant protein, three regions of protection of the *nadA* promoter are visible. Two appear on addition of 30 nM of NadR protein: the first (OpI) spanning from -139 to -119 and the second (OpII) spanning from -15 to +7 and, therefore, within regions of the GPR and P5 probes that were previously shown to be bound by the MC58 extracts as well as a third region (OpIII) spanning the TAAA tract from -55 to -85. EMSA analysis confirmed that NadR exhibits high affinity for the GPR and P5 operator regions and exhibits a lower affinity for the TAAA tract. These observations were supported by EMSA analysis with a probe spanning the entire P_{nadA} promoter as three differential protein-DNA complexes were formed, most likely following sequential binding of the protein to the operators located within the P_{nadA} probe (Figure 11B).

To confirm that NadR is the GPR-binding factor, we generated a deletion *nadR* mutant by substituting the gene with an antibiotic resistance marker. Cell extracts derived from the *N. meningitidis* Δ1843 mutant no longer possessed binding activity towards the GPR probe (Figure 12A).

From this analysis we conclude that NadR encodes the GPR-binding factor that binds to three operators; two high affinity operators OpI and OpII within the distal GPR region and overlapping the *nadA* promoter, respectively, and a lower affinity operator OpIII which spans the TAAA repeat tract.

Figure 11

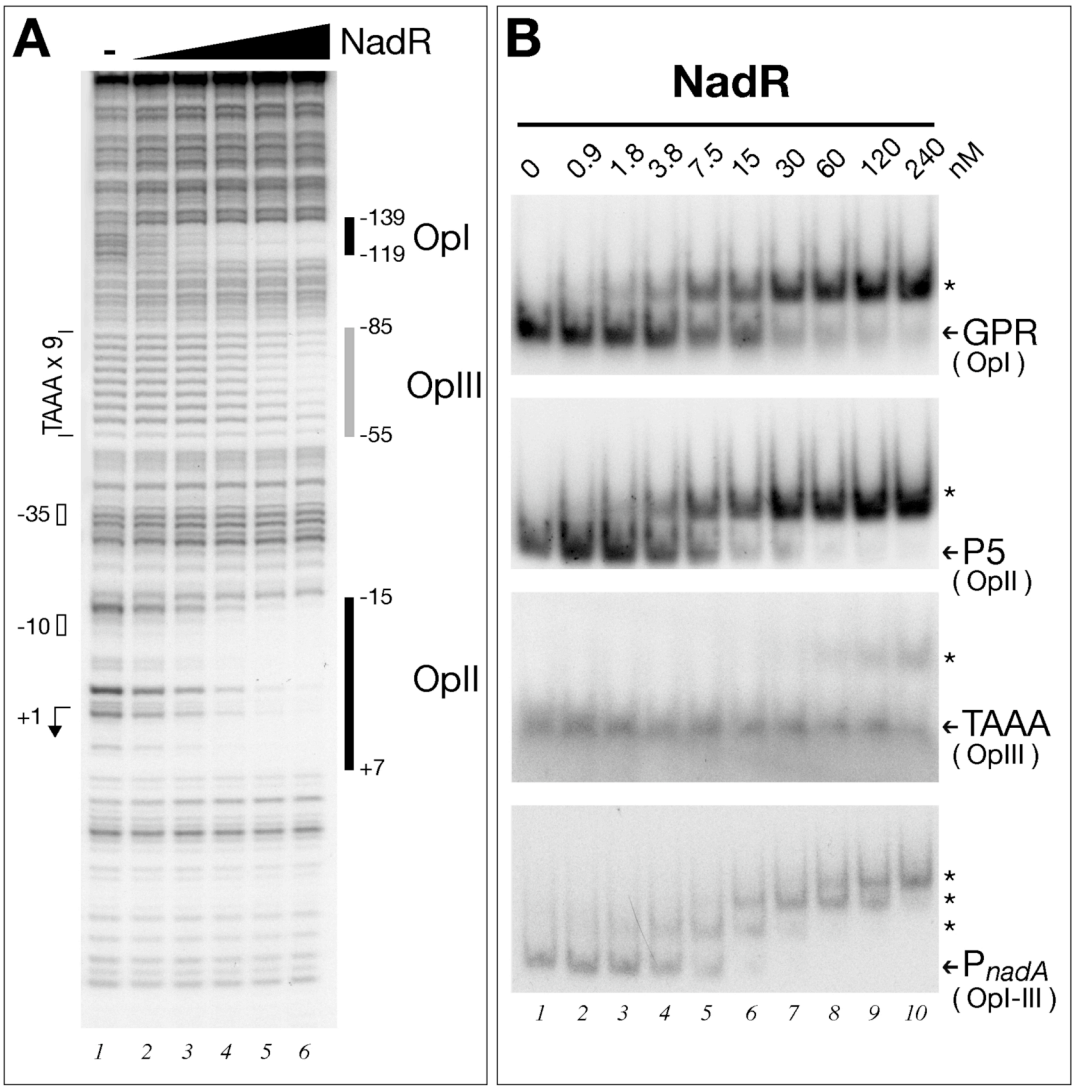


Figure 11: The NadR repressor binds specifically to three operators in the *nadA* promoter. (A) DNase I footprinting analysis with purified NadR on the *nadA* promoter with 9 repeats. The NadR protected regions are indicated (OpI-III) and numbers represent the nucleotide positions with respect to the transcriptional start site. The size of protected regions ranges from 20 bp (OpI and OpII), and 30 bp (OpIII), a size compatible with the binding of a protein dimer. Binding reactions contained 40 fmoles of probe radioactively labelled at one extremity and 0, 7.5, 15, 30, 60, 120 nM of NadR purified dimer (lanes 1-6, respectively). (B) EMSA with radioactively labelled GPR, TAAA and P5 probes containing the individual OpI, OpIII and OpII operators, respectively, or the entire P2 *nadA* promoter spanning from -170 to +79 with increasing concentrations of recombinant NadR protein as indicated. The retarded migration of protein DNA complexes are indicated with asterisks.

1.19 NadR represses NadA expression

To further study the role of NadR in regulating NadA expression, and its possible involvement in mediating differential expression from phase variant promoters, we generated isogenic knockouts in five representative strains bearing different numbers of tetranucleotide repeat in their *nadA* promoter which correlate to high (8 repeats, 5/99) and low (9 repeats, MC58), as well as three intermediary (5 repeats, BZ83; 6 repeats, ISS838, and 12 repeats, 961-5945) levels of NadA expression. We evaluated by Western Blot the NadA and NadR expression level in the wild type and Δ1843 meningococcal strains. The wild type strains showed, as expected, levels of NadA expression that can be associated with transcript levels of the *nadA* phase variant promoter they bear, and NadR was constitutively expressed in each strain (Figure 12B, WT). Each of the knockout strains exhibits higher levels of NadA expression than their respective wild type strain indicating that NadR represses *nadA* expression in each strain (Figure 12B WT vs Δ1843). Surprisingly, the mutation of NadR results in almost equivalent levels of NadA between the knockout strains, although the 5/99-Δ1843 and BZ-Δ1843 still exhibit slightly higher NadA expression. This suggests that NadR, although expressed to the same level, has a different repressive activity on the *nadA* gene transcription in each strain and this may depend on the number of repeats in the different phase variant promoters i.e. NadR does not efficiently repress the 8x promoter of 5/99 but very efficiently represses the 9x promoter of MC58.

Figure 12

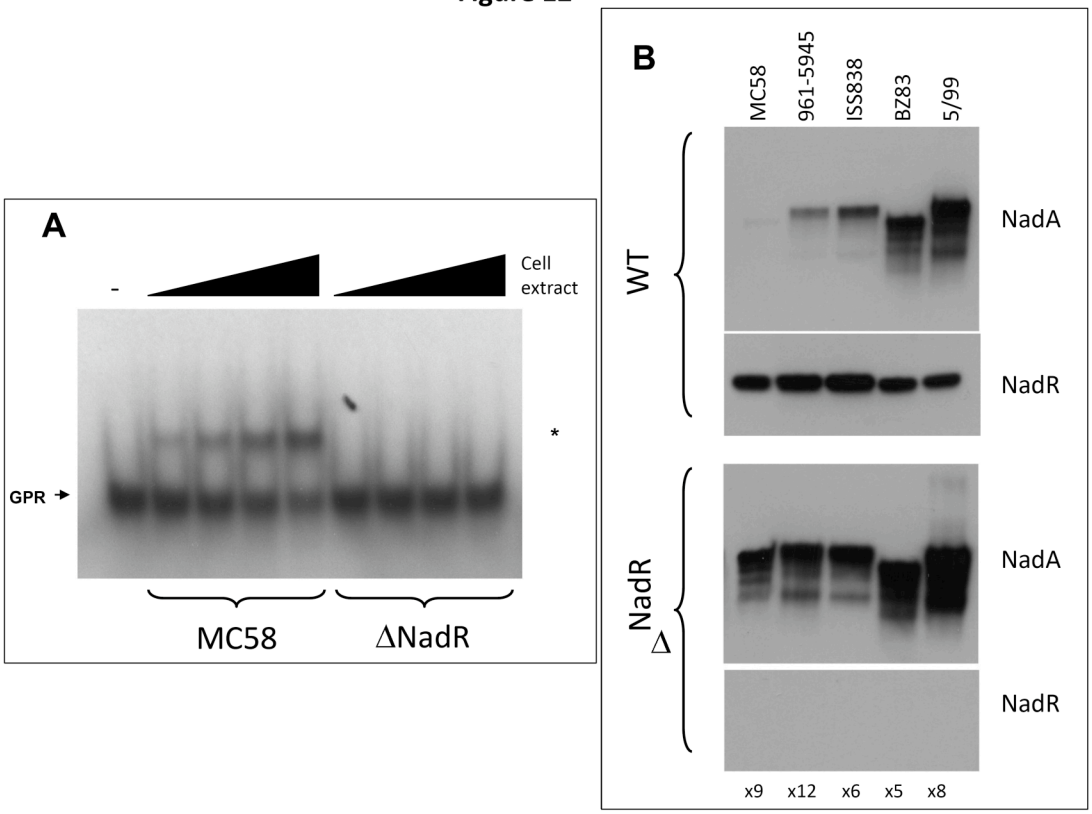


Figure 12: NadR represses NadA expression. (A) EMSA assay performed with MC58 and Δ1843 cell extract on GPR probe showing the lack of binding activity in absence of NadR. (B) Western Blot analysis of the level of expression of NadA and NadR in wild type strains 5/99, BZ83, ISS838, 961-5945 and MC58 carrying *nadA* promoters with 8, 5, 6, 12, and 9 repeats respectively, and their NadR null mutant derivatives. Cells were recovered from overnight culture on plates and 5 μg of total protein were loaded on SDS-PAGE, blotted and stained with anti-NadA or anti-NadR polyclonal antiserum.

1.20 NadR represses to a different extent different phase variant promoters

To further test the latter hypothesis and to rule out effects due to strain differences, we deleted the *nadR* gene in the isogenic MC58 strains carrying high (x8), medium (x6) and low (x9) promoter variants and measured the steady state levels of transcription from the promoters at log and stationary growth phase in the presence or absence of the NadR regulator. The results in Figure 13 confirm that in the mutant (Δ1843) all three promoters are derepressed and, interestingly, little or no variation in transcript levels between the phase variants is observed, suggesting that in the absence of NadR the mechanism of transcriptional control exerted by variable number of repeats is alleviated or negligible. It is worth noting, however, that the maximum level of transcription in exponentially growing cells is observed from the promoter variant with 8 repeats, in agreement with higher NadA expression in 5/99-Δ1843, suggesting that NadR is not the sole modulator of phase variable promoter activity and that there is another factor which may establish differential RNAP contacts to modulate transcription.

Furthermore, we also measured the transcript level of the PΔ promoter, which lacks the TAAA tract and also no longer binds IHF, in the wild type and Δ1843 backgrounds and results indicate that NadR does not efficiently repress this mutant promoter (lanes 9 and 10 versus 1 and 2) and implicates a major role for IHF in efficient NadR-mediated repression of the *nadA* promoter.

Figure 13

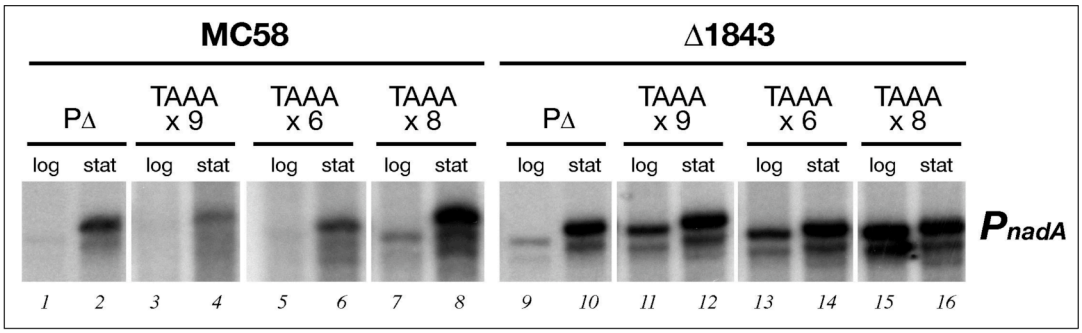


Figure 13: The NadR repressor contributes to phase variable expression. Transcription of phase variant promoters with 0, 9, 6, and 8 repeats, in the MC58 and NadR null mutant backgrounds. Total RNA was prepared from cultures of strains MC-PΔ, MC-P2(x9), MC-P2(x6), MC-P2(x8), Δ1843-PΔ, Δ1843-P2(x9), Δ1843-P2(x6), and Δ1843-P2(x8), grown to mid-log and stationary growth phase. Quantitative primer extension was performed as described in materials and methods.

1.21 4-hydroxyphenylacetic acid (4HPA) interacts with NadR and derepresses NadA expression

The MarR family of proteins regulates a wide variety of biological processes including resistance to antibiotics and antimicrobial agents, virulence and environmental sensing of aromatic compounds (Wilkinson and Grove 2006) (Ellison and Miller 2006). They respond to small inducer molecules which attenuate the ability of MarR homodimers to bind their cognate DNA sequences (Wilkinson and Grove 2006), and are often the molecular substrates for the efflux pumps or metabolic pathways that are repressed by this family of regulators. We set about identifying a small molecule inducer, which may regulate NadR-mediated repression of NadA expression in meningococcus. We assessed broad-specificity inducers such as salicylic acid, which have been shown to be active against many members of this family, and also functionally relevant molecules such as long-chain fatty acids, which are the substrate for the regulated efflux pump of the gonococcal NadR homologue FarR (Lee, Rouquette-Loughlin et al. 2003) with no success. However, we noticed that immediately downstream of the *nadR* gene is an ORF which encodes a putative flavoprotein oxidoreductase with 42% amino acid identity to the small subunit of 4-hydroxyphenylacetic acid 3-hydroxylase. In addition, the closest BLAST neighbour of NadR in the MarR family of repressors is the HpaR protein (50% identity), which represses the 4-hydroxyphenylacetic acid (4HPA) catabolic pathway in *E. coli.* Moreover, it is responsive to the 4HPA substrate of the pathway, which binds to the repressor and induces expression of the catabolic genes (Galan, Kolb et al. 2003). We therefore, assessed whether the 4HPA molecule could act as putative inducer of NadA expression in vivo. Addition of 1 mM or 5 mM 4HPA (Figure 14A) to cultures of MC58 significantly induced NadA expression. No induction could be detected in cultures of the Δ1843 mutant, indicating that the 4HPA molecule

induced a NadR-mediated derepression of NadA expression. Then we further analyse transcript level of *nadA* after treatment of MC58 cultures with increasing concentration of 4HPA. As shown in figure 14 B *nadA* transcript increases upon addition of 200µM of the molecule and reaches the maximum at 1 mM after only 10 minutes of treatment (Figure 14 B lanes 3, 4), suggesting that the induction is directly at the level of promoter derepression. To confirm that the observed increases in NadA expression could represent a direct interaction of the inducer with NadR, the ability of the compound to dissociate purified recombinant NadR from the high affinity operator OpI was assessed by EMSA. The 4HPA compound was found to attenuate the binding activity of the NadR regulator to the GPR probe in vitro (Figure 14C). Furthermore, addition of 1 mM 4HPA to crude cell extracts containing the native NadR meningococcal protein resulted in complete inhibition of retardation of the GPR probe in EMSA (data not shown), suggesting that the recombinant and native NadR proteins respond in vitro similarly to the compound. These data suggest that the 4HPA could be a ligand of the NadR repressor and interaction of the ligand with the protein attenuates the DNA-binding activity of the molecule for its specific operators and results in derepression or induction in vivo of the *nadA* promoter.

Figure 14

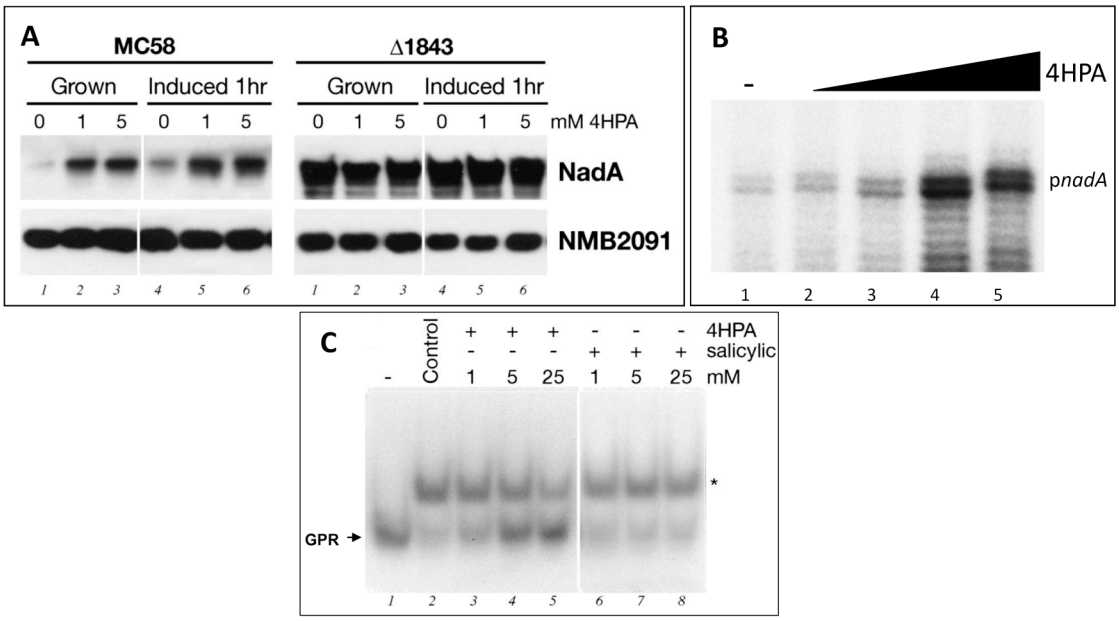


Figure 14 (A) Induction of expression of NadA by addition of a small molecule ligand 4-hidroxy phenyl acetic acid (4HPA). Broth cultures of MC58 or Δ1843 were grown to OD_{600} of 0.24 without (lane 1) or with 1mM or 5mM (lane 2 and 3) 4HPA; or to OD_{600} of 0.24 and then incubated with 0, 1 or 5mM 4HPA (lanes 4-6) added for 1 h. Cells were harvested and 5 μg of total protein from each culture was subjected to SDS-PAGE and Western Blot analysis with anti-NadA or anti-NMB2091 antibodies as negative control. (B) Primer extension showing *nadA* transcript level from total RNA of MC58 strain grown at OD_{600} of 0.5 and treated for 10 minutes with 0, 0.04, 0.2, 1 and 5 mM of 4HPA (lanes 1-5 respectively). (C) EMSA assays demonstrating dissociation of NadR from OpI operator in the GPR probe in vitro following the addition of 4HPA (lanes 3-5) but not the broadly acting salicylic acid ligand (lanes 6-8).

Discussion

Host-pathogen interaction is a dynamic process that can lead to different outcomes such as the clearance of the pathogen, the establishment of a disease or a sort of equilibrium leading to commensalism. The factors that lead *N. meningitidis* to establish a productive infection, switching from commensal to pathogenic, thought to be dependent on both the host and the pathogen are still poorly understood. For these reasons, a better understanding of the causes and mechanism that mediate the expression of proteins involved in the interaction with host tissues is needed, both for predicting the effectiveness of a vaccine which contains these proteins, as well as for characterizing at the molecular level, novel strategies in the ever changing fight between pathogens and their host.

In this scenario *N. meningitidis* has to arrange in concert the expression of a variety of different genes in order to adapt and survive in the different tissues during an infection of the human host. At the same time the bacteria must also escape the host immune response, which targets mostly the same structures used by the meningococcus to interact with the host. This issue is addressed by the high redundancy and variability in the expression of surface structure and by the ability to acquire genes from other bacteria species.

The low GC content, with respect to the average of the genome, and the presence of a repeated sequence (TCAGAC) flanking the *nadA* gene, suggest it was acquired by horizontal transfer (Comanducci, Bambini et al. 2002). Another repeated tract present in the *nadA* locus (TAAA tetranucleotide repeated tract upstream of the promoter), has been found to be responsible for its phase variable expression (Martin, Makepeace et al. 2005).

Phase variation is the adaptive process by which bacteria undergo frequent and reversible phenotypic changes resulting from genetic alterations in specific loci of their

genomes and this process is crucial for the survival of pathogens and commensals in hostile and ever-changing host environments. *N. meningitidis* has an unprecedented potential for generating genetic diversity through slipped strand mispairing of simple sequence repeats, as its genome contains over 100 repeat associated genes (Saunders, Jeffries et al. 2000; Snyder, Butcher et al. 2001). The way in which genes are affected by variation in the number of repeats is largely thought to occur through biphasic on/off translational control due to frameshifting within the ORFs of coding regions. Recently the on/off switching of methyltransferase genes has been shown to co-ordinate expression of a phase-variable regulon of genes or "phasevarions" via differential methylation of the genome (Srikhanta, Maguire et al. 2005) (Srikhanta, Dowideit et al. 2009). The role of simple sequence repeats in intergenic regions in modulating phase variable expression, although frequently found, are less easy to predict. However, differential spacing due to repeated tracts between the core promoter elements modulating multi-phasic expression by affecting RNAP sigma factor binding has been frequently reported (Sarkari, Pandit et al. 1994) (Carson, Stone et al. 2000) (van Ham, van Alphen et al. 1993) (van der Ende, Hopman et al. 2000), as well as some documented examples where repeats in 5'UTR (Dawid, Barenkamp et al. 1999) (Lafontaine, Wagner et al. 2001) and distally upstream (Martin, van de Ven et al. 2003) (Dawid, Barenkamp et al. 1999) (Puopolo and Madoff 2003) (Liu, Panangala et al. 2002) of promoters have been shown to affect expression through unknown mechanisms.

In this study, we dissect the cis- and trans-acting elements as well as environmental factors that control transcriptional regulation of the *nadA* promoter in order to elucidate the mechanism by which sequence repeats distally upstream of the P*nadA* promoter controls its activity. We describe a complex promoter architecture in which spontaneous changes in the number of simple sequence repeats in a tract between the most distal

regulatory regions and the core promoter can alter the promoter activity and lead to phase variable expression. We have shown that the NadR repressor is the major contributor to the phase variable expression levels of the promoter as it binds to two high affinity operators flanking the repeats. One operator overlaps the -10 region of the promoter and the transcriptional start site, and therefore, binding of NadR is consistent with its function as a repressor through sterically hindering RNAP access to the promoter. The other high affinity operator is on the distal upstream side of the phase variable repeat in a cis-acting region that we call the GPR, which is functionally active in repressing the promoter despite its distal location (Figure 8). We have identified a single IHF binding site that is located between these two high affinity operators and we show that IHF binding to this promoter is necessary for efficient NadR-mediated repression of P_{nadA}. The IHF binding site comprises some sequence upstream of the TAAA as well as part of the TAAA tract itself. If the TAAA tract is removed, the protein no longer binds the mutant promoter (Figure 7). However, we found that IHF binding is unaffected by the number of TAAA repeats, but rather the number of repeats may change the spacing of the DNA on the upstream and downstream flanking regions of the tract and, therefore, may influence the localisation, and possibly the orientation of proteins that bind to the operators. The ability of IHF to bend DNA may facilitate the looping of the DNA of the *nadA* promoter and bring the GPR element proximal to the core promoter elements. A looping mechanism (Figure 15) would explain the function of such a distal operator in repression of transcription, possibly through interactions of dimers present on spatially proximal operators which lock the promoter to RNAP similar to the mechanism described for the *lac* operon (Oehler, Eismann et al. 1990) (Lewis, Chang et al. 1996).

1.22 Three-dimensional MODEL

Figure 15

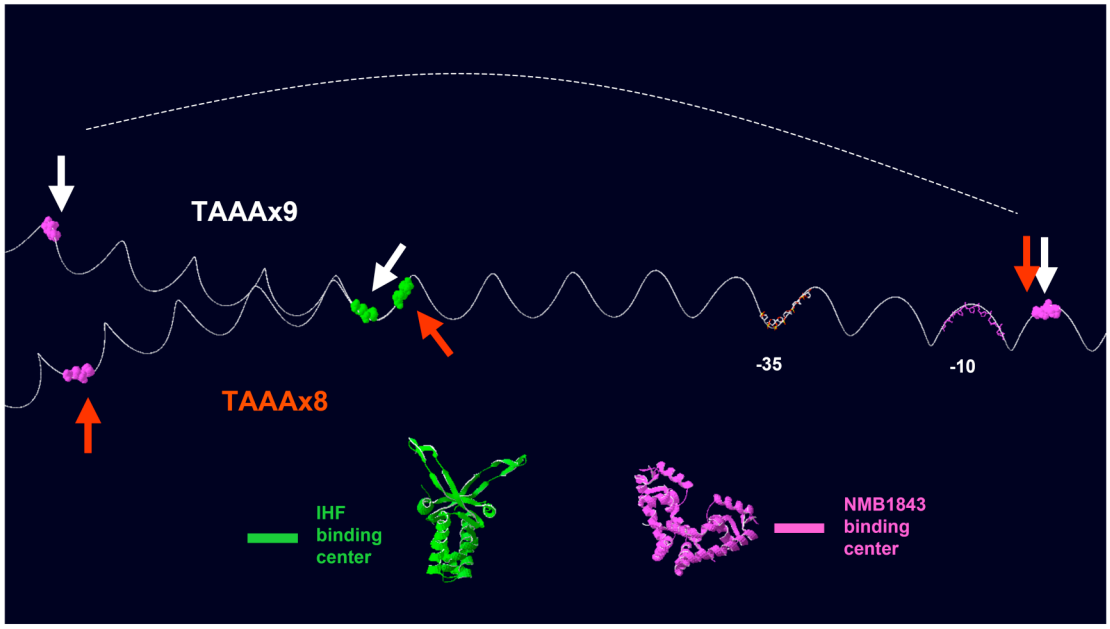


Figure 15: Three-dimensional structure of *nadA* promoter region. The spatial conformation of the DNA filament is predicted on the basis of its sequence composition (Predictor and Swiss-pdbViewer). Forward strands of two *nadA* phase variants promoters (x9 and x8 TAAA repeats) are shown, superimposed from their 3' ends. The -35 and -10 examers are indicated and two nucleotides corresponding to the centre of the binding sites for IHF (green) and NMB1843 (purple), identified by footprinting, are shown in Space-Fill mode and depicted with either white or red arrows (for phase variant promoters with 9 and 8 repeats respectively).

However, in the completely derepressed form there are still significant, albeit marginal differences in the promoter strength of variants with different numbers of repeats. The alpha-subunit of RNAP binds to the distal GPR regulatory region and also immediately upstream of the core promoter overlapping the TAAA tract which may function as UP-like elements. We propose a model in which differential distancing between the *nadR* operators and the contact points of RNAP result in optimal or suboptimal configuration of the protein complexes and, therefore, result in more or less efficient repression mediated by NadR and/or more or less cis-enhancement of RNAP activity on the basal promoter strength (Figure 16).

1.23 Regulation MODEL

Figure 16

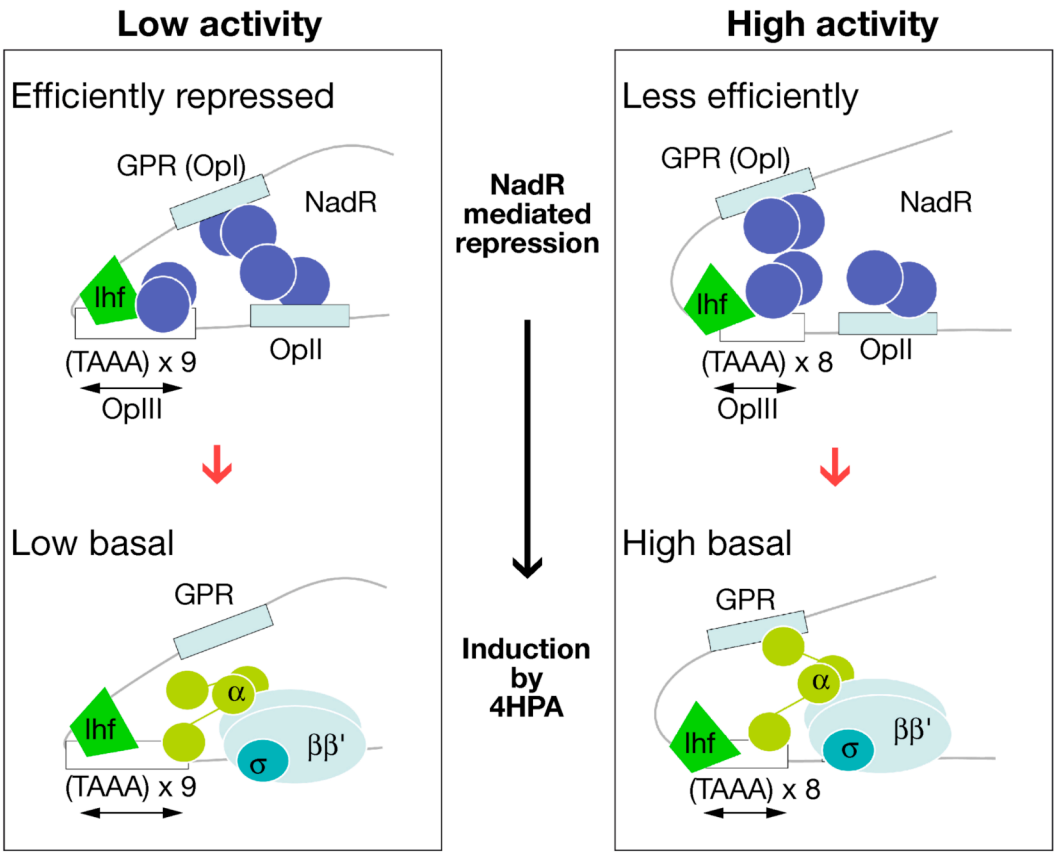


Figure 16: Model of regulation of NadA promoter: Two promoter variants with 9 and 8 repeats representing low activity and high activity promoter phase variants, respectively, highlighting the ability of NadR to efficiently or less efficiently repress the promoters (top panels) and NadR-independent effects 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 RNAP contact points resulting from the different number of repeats.

Interestingly, in Pu promoter of *P. putida*, two distinct UP elements, located close to the core promoter (-79) and distally upstream (-104), interact with RNAP α-subunits enhancing gene expression (Macchi, Montesissa et al. 2003). This interaction is modulated by IHF that allow the interchangeable positioning of the two α-subunits over the two UP-elements (Macchi, Montesissa et al. 2003) (Bertoni, Fujita et al. 1998). This scenario resembles in part what we observe with α-subunit interaction over *nadA* promoter.

The gonococcal homologue of NadR is FarR, which has been characterised in gonococcus as a repressor of the FarAB fatty acid resistance efflux pump (Lee, Rouquette-Loughlin et al. 2003). FarR binds two distal operators on the *farAB* promoter (located similarly to OpI and OpII in the *nadA* promoter) and represses transcription in an IHF dependent way. It would appear from deletion analysis of the *nadA* promoter that all regulatory elements necessary for control of *nadA* expression were horizontally transferred together with the *nadA* gene, as the direct repeat delineating the border of the transferred DNA is at -193. The intriguing question is then how the *nadA* locus, which is present in only a fraction of strains, through a horizontal transfer event, adopted such a complex regulatory mechanism pre-existing in *Neisseria*. The *nadR* gene is well conserved in other *Neisseria* spp such as *N .mucosa*, *N. cinerea, N. lactamica, N. subflavia* and *N. flavescens* and, therefore, must respond to signals in the ecological niches of all these species.

The NadR protein is a member of the MarR ligand-responsive transcriptional regulators and the majority of MarR family members are regulated by the non-covalent binding of low molecular weight ligands. These signalling molecules regulate the activity of the regulators. In this study, we have identified a putative ligand, 4-hydroxyphenylacetic acid, which is able to relieve the DNA binding activity of NadR, thus derepressing or

inducing NadA expression. This molecule is a catabolite of aromatic amino acids and it is secreted in human saliva (Takahama, Hirota et al. 2003) (and also urine), suggesting that the inducer is present in the oropharynx and NadA may be induced in the mucosal niche, which is bathed in saliva.

The 4HPA molecule is a catabolite of the aromatic amino acids, tyrosine and phenylalanine. Two groups of bacteria, the soil inhabitants such as *P. putida* and the enteric bacteria such as *E. coli* contain pathways for the breakdown of these amino acids to succinate and pyruvate (Diaz, Ferrandez et al. 2001). However, such a pathway is not present in the meningococcal genome (Tettelin, Saunders et al. 2000). Nevertheless, *nadR* is present in a operon-like organization with 2 downstream genes one of which shows significant homology to the HpaC small subunit of a hydroxylase involved in the conversion of 4HPA to a less toxic form (3,4-dihydroxyphenylacetic acid). It is unclear whether this operon may be involved in the utilisation of 4HPA inducer in some way in meningococcus, or whether it is the remnant of a partial catabolic pathway that was acquired horizontally and the 4HPA molecule simply acts as a signal inducing the expression of the adhesin, which is necessary for colonization and invasion of the mucosa.

Phase variation functions as an adaptive strategy generating spontaneous diverse sub-populations of the bacterium which may be beneficial in adapting to different microenvironments within the human host during the course of a natural infection. However, in the case of *nadA* gene regulation, this type of mechanism is bi-functional, in that the major mediation of phase variable expression levels of NadA is through repression by NadR protein in the absence of the correct inducer signal. Subpopulations expressing low levels of NadA through promoter phase variations still have the potential to respond to the correct niche signal, such as 4HPA, and express high levels under certain environmental or niche-specific conditions. Such variation will have an

impact on the interaction with the host tissues, as well as escaping immune responses. Simple sequence repeats have been identified in distal promoter regions of known or potential virulence factors in other pathogens including *Helicobacter pylori* (Saunders, Peden et al. 1998), *H. Influenza* (Dawid, Barenkamp et al. 1999) (Parkhill, Achtman et al. 2000), *Moraxella catarrhalis* (Linton, Karlyshev et al. 2001), Group B *Streptococcus* (Puopolo and Madoff 2003) and pathogenic *Mycoplasma* (Liu, Panangala et al. 2002) some of which have been shown to control phase variable expression through unknown mechanisms. This suggests that complex regulatory mechanisms such as what we have elucidated for NadA involving stochastic variations and environmentally-responsive transcription factors may be widely used by pathogens. Elucidating these mechanisms is important for our understanding of the intimate and complex relationship between the host and disease-causing organisms.

Materials and Methods

1.24 Bacterial strains and culture conditions

The *N. meningitidis* strains used in this study are all listed in Table II. *N. meningitidis* strains were routinely cultured in GC-based (Difco) agar medium supplemented with Kellogg's supplement I (Kellogg *et al*., 1963) at 37°C in a 5% CO_2-95% air atmosphere at 95% humidity. Strains were stocked in 10% skim milk and stored at -80°C. For liquid cultures, *N. meningitidis* strains were grown overnight on solid medium, re-suspended in GC broth to an optical density at 600 nm (OD600) of 1, and inoculated with a 1:20 dilution into GC broth supplemented with Kellogg's supplement I, 12.5 μM $Fe(NO_3)_3$ or Mueller Hinton (MH) (Sigma, St. Louis, MO) containing 0,25% glucose and, when required, erythromycin and/or chloramphenicol added to achieve final concentrations of 5 μg ml^{-1}. For transformation by naturally competent *N. meningitidis*, four to five single colonies of a freshly grown overnight culture were re-suspended in 20 μl of PBS, spotted onto GC plates, and 5–10 μg of linearized plasmid DNA was added, allowed to dry and incubated for 6–8 h at 37°C. Transformants were then selected on plates containing erythromycin (5 μg ml-1) and/or chloramphenicol (5 μg ml-1), and single colonies were re-streaked on selective media for further analysis. Transformants were re-suspended in 50 μl of distilled water, placed in a boiling water bath for 5 min and centrifuged in a bench top centrifuge for 5 min at 8000 *g*. One microlitre of the sample was used as template for PCR analysis for correct insertion by a double homologous recombination event, and *nadA* promoter regions were amplified and sequenced to verify that no phase variation had occurred during manipulations. *E. coli* DH5-α (Hanahan 1983) and BL21(DE3) (Studier and Moffatt 1986) cultures were grown in

Luria–Bertani medium, and when required, ampicillin chloramphenicol and/or IPTG were added to achieve final concentrations of 100, 20 μg ml-1 and 1mM respectively.

1.25 DNA techniques.

DNA manipulations were carried out routinely as described by Sambrook, Fritsch et al. 1989. Small- and large-scale plasmid DNA preparations were carried out with a QIAprep Spin Mini kit and Plasmid Midi kit (QIAGEN) according to the manufacturer's instructions. DNA fragments or PCR-amplified products were purified from agarose gels with QiaEX DNA purification kit (QIAGEN). PCR was performed in a Perkin-Elmer 2400 thermal cycler with Platinum Taq polymerase (Invitrogen). One microlitre of each reaction mixture contained 10–50 ng of chromosomal DNA or 1 μl bacterial sample (see above), 100 pmols of the required primers and 200 μM concentration of each deoxynucleotide in a volume of 100 μl of 1 x PCR buffer containing MgCl2 (Invitrogen). After the initial denaturing step at 95°C for 5 min, 30 cycles of denaturing at 95°C, annealing at the appropriate temperatures for the specific primers and elongation at 72°C were carried out. DNA fragments were routinely sequenced according to the dideoxy-chain termination method (Sanger, Air et al. 1977) by using [α-32P]-dATP (NEN, Perkin Elmer) and a T7 sequencing kit (Pharmacia).

1.26 Construction of *nadA* promoter fusions

In order to analyze cis-elements involved in regulation of *nadA* expression a series of plasmids carrying *nadA* promoters fused to the *gfp* gene were constructed, which could be assayed in *E. coli* and also used for allelic exchange in *N. meningitidis* strains at a chromosomal location between two converging open reading frames (ORFs), NMB1074 and NMB1075. A plasmid consisting of a promoterless *gfp* gene and the *ermAM* erythromycin-resistance genes flanked by upstream and downstream regions for

allelic replacement was generated (pGFP, (Ieva, Roncarati et al. 2008)). A series of 5' deletion variants of *nadA* promoter were generated by PCR amplification from the genome of MC58 using primers Nad-N1, Nad-N2, Nad-N3, Nad-N4 and Nad-N5 as forwards and Nad-Sp as reverse, generating P1, P2, P3, P4 and P5 promoter deletion fragments respectively, spanning from -273, -170, -108, -49, and -9, respectively, to +79 with respect to the transcriptional start site. The 5 promoter fragments were cloned using NsiI and SphI sites, introduced by primers design into the pGFP plasmid and used for transformation of MC58 generating the MC-P1, MC-P2, MC-P3, MC-P4, amd MC-P5 strains respectively. (Table II and Figure 8). Phase variant *nadA* promoters with different repeat numbers, from 4 to 13, were PCR amplified with the Nad-N2/Nad-Sp primer pair using as a template genomic DNA from different clinical isolates with the appropriate number of repeats as major clone kindly donated by Maurizio Comanducci and Stefania Bambini. All promoters fragments generated, (spanning from -170 to +79 with respect to the +1 in MC58) were then cloned as NsiI- SphI fragments into the pGFP plasmid and used for transformation of MC58 generating the MC-P2x4, MC-P2x5, MC-P2x6, MC-P2x7, MC-P2x8, MC-P2x9, MC-P2x10, MC-P2x11, MC-P2x12, MC-P2x13 strains respectively. (Table II and Figure 5). The PΔ *nadA* promoter variant, lacking the TAAA tetranucleotide repeat, was generated by PCR amplificaion of regions upstream and downstream of the TAAA repeated tract, using primer pairs Nad-N2/n85-50R and n85-50F/Nad-Sp. Then, in a second round of PCR, the respective upstream and downstream fragments, which contain regions of overlap due to the primer design, were used in a self-priming PCR amplification for 5 cycles, and then the corresponding united fragments were amplified using the external primers Nad-N2/Nad-Sp, and cloned into pGFP. The pGFP-PΔ plasmid was used for the transformation of MC58 generating MC-PΔ, containing a *nadA* promoter fusion in which the TAAA repeated tract was substituted with an *EcoR*I site (Table II and Figure 8).

1.27 Construction of knockouts

In order to knockout the NMB1843 gene in the *Neisseria* background, the pΔ1843ko::Cm plasmids was constructed. Upstream and downstream flanking regions of the NMB1843 gene were amplified by PCR with the 1843-1/1843-2b and 1843-3b/1843-4 primers respectively. Then in a second round of PCR the respective upstream and downstream fragments, which contain regions of overlap due to the design of the primers, were used in a self-priming PCR amplification for 5 cycles, and then the corresponding united fragment was amplified using the external 1843-1/1843-4 primers. This product was cloned into the pGEM-T (Promega) vector and a chloramphenicol cassette from pDT2548 (Wang and Taylor 1990) was inserted into the unique *Bam*HI site, between the flanking regions, generating pΔ1843ko::Cm. The plasmid was then linearised and used for transformation of the meningococcal strains to make the respective NMB1843 knockout mutants (Table II). The correct double homologous recombination event resulting on the knockout of the genes was verified by PCR.

1.28 Expression and purification of the *E.coli* RNA polymerase α-subunit and *N. meningitidis* NMB1843 protein.

The *NMB1843* gene was amplified from the MC58 genome with the 1843-F/1843-R primer pair and cloned as a 448 bp *Nde*I-*Bam*HI fragment into the pET15b expression plasmid (Invitrogen) generating pET15-1843 which was subsequently transformed into the *E. coli* strain BL21(DE3) for protein expression. For alpha subunit expression, plasmid pHTT7f1-NHα (Tang, Severinov et al. 1995) was transformed into *E. coli* strain BL21(DE3).. From an overnight culture of the BL21(DE3)(pET15-1843) and BL21(DE3)(pHTT7f1-NHa) strains, 200 ml of Luria-Bertani medium was inoculated

and grown to an OD_{600} of 0.5, and expression of the recombinant NMB1843 or *E.coli* RNApol α-subunit proteins containing an N-terminal histidine tag was induced by the addition of 1 mM IPTG (isopropyl-D-thiogalactopyranoside) and further incubation for 3 h. The proteins were purified from the harvested cells by Ni-nitrilotriacetic acid (QIAGEN) affinity chromatography under nondenaturing conditions according to the manufacturer's instructions. The purified protein preparation was then diluted to 1 mg/ml and dialyzed overnight in 1 x Binding Buffer (20 mM Tris-HCl pH 8, 50 mM KCL, 10 mM $MgCl_2$, 01% NP40) containing 10% glycerol and then again overnight in 1x Binding buffer containing 50% glycerol. The purity of the proteins was estimated to be greater than 98% by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of the proteins in this preparation was determined by using the Bradford colorimetric assay (Bio-Rad), and the protein was aliquoted and stored at -80°C. To generate anti-NMB1843 antibodies, 6-week-old female CD1 mice (Charles River Laboratories) were immunized with 20 μg of NadR protein given intraperitoneally, together with complete Freund's adjuvant in three doses (day 1, 21 and 35). Bleed-out samples were taken on day 49 and used for Western blot analysis.

1.29 Western blot analysis

N. meningitidis colonies from overnight plate cultures were either resuspended in PBS until OD_{600} of 1, or grown to logarithmic phase (OD of 0.5, ca. 2 hours incubation) and stationary phase (OD ≥ 0.9 ca. 5 hours incubation) from a starter inoculum of OD 0.05. Samples of 1-2 ml were harvested and normalised in 1xSDS-PAGE loading buffer (50 mM Tris Cl pH 6.8, 2.5 % SDS, 0.1 % Bromophenol Blue, 10% glycerol, 5% B-mercapto-Ethanol, 50 mM DTT) to a relative OD of 5. For Western blot analysis, 10 μg of each total protein sample in 1 x 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 4C° by agitation in blocking solution (10% skimmed milk, 0.05% Tween-20, in PBS) and incubated for 90 min at 37°C with anti-NadA or anti-NMB1843 polyclonal sera) in 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) respectively, in blocking solution for 1 hour and the resulting signal was detected using the Supersignal West Pico chemiluminescent substrate (Pierce).

1.30 Co-over expression of alpha subunit in *E. coli*

In order to allow co-expression in *E. coli* of *nadA* promoter fusions with pLAW2 (overexpressing RNAP α-subunit) and pLAW2Δ256 (overexpressing the N-terminus of α-subunit), pXG-1 plasmid (Urban and Vogel 2007), harbouring pSC101 origin of replication and a cloramphenicol resistance cassette, was used simply substituting the 181 bp AatII/NheI fragment containing the PLtetO-1 promoter with different *nadA* promoter phase variants amplified with nad-Aa2/nadNh primer pairs (Table I), generating *nadA-gfp* translational fusions.

To evaluate the role of RNAP alpha-subunit in regulation of transcription from the *nadA* promoter, we co-transform pLAW2 or pLAW2Δ256 with pXG harbouring *nadA*-gfp fusions in *E. coli*. After liquid growth to an OD_{600} of 0.5 in presence of 1 mM IPTG for induction of α expression, GFP fluorescence was measured in 48 well plates using TECAN Infinite 200 with excitation wavelength of 460 nm and an emission of 510 nm. Experiments were performed in triplicate.

1.31 Crude extract preparation, DNA affinity purification of GPR-binding protein and Electrophoretic mobility shift assay (EMSA)

N. meningitidis strains were growth until OD_{600} of 0.5 as described above, then 100 ml were harvested and resuspended in 10 ml of PBS, sonicated at maximum power (20 impulse of 0,8 second each) for 5 times at +4 °C and the cell debris was removed by centrifugation at 8,000xg for 15 min. The soluble fraction was then filtered using a 0.2 µm membrane and stored at 4 °C. Proteins concentration in the sample was determined using the Bradford colorimetric assay.

For the characterization of the binding activity in the cell extract, it was precipitated, where indicated, with ammonium sulphate salts by adding, from a saturated solution, the amount to reach 20, 40, 60 and 70% concentration. Cell extracts were also incubated at different temperatures (40, 60 and 100°C) for 30 minutes or with 5 µg of Trypsin (Promega) or Proteinase K (Gibco BRL) for 30 minutes or 2 hours as indicated.

For purification of GPR-binding protein from MC58 crude extract a probe spanning from -170 to -116 with respect to the MC58 *nadA* promoter was amplified using a 5' biotinylated forward primer Bio-nadN2 (Invitrogen custom primers) and gpr-R reverse primer. 25 pmoles of the fragment were incubated with 1.3 ml of MC58 crude extract (0.6µg/µl) in presence of 100µg of salmon sperm DNA and 500µg bovine serum albumin (BSA) to block non specific interactions for 20 minutes at room temperature with gentle rotation. The mixture was then added to 2,5 mg of Dynabeads M-280 streptavidin (Invitrogen), previously washed 4 times with 250 µl PBS, and incubated for 20 minutes at room temperature with gentle rotation. The tube was then placed on a magnet for 2 minutes for magnetic separation of the beads and after 3 washes with PBS, proteins bound to biotinylated GPR were eluted in 400µl of 1M NaCl. The sample was

then dialyzed over night against H_2O at 4 C°. All fractions were then analysed for binding activity using EMSA assay, and eluted fraction was further analyzed using MALDI TOF.

For gel shift experiments, a probe corresponding to the GPR element and spanning from -170 to -116 with respect to the MC58 *nadA* promoter was amplified using nad-N2/gpr-R, a probe corresponding to the P5 promoter fragment spanning -9 to +81 was amplified with NadN5/Nad-Sp, a probe corresponding to P2 promoter fragment spanning -170 to +81 was amplified with nad-N2/ Nad-Sp, a probe corresponding to 6 TAAA repeats using primers IHF-Lex and IHF-Rex2 annealed and a probe corresponding to an unrelated intergenic region Pcon (157 bp between NMB2073 and NMB2074), was amplified using SR-F and SR-R. 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). The unincorporated radioactive nucleotides were removed using the TE-10 chromaspin columns (Clontech). For each binding reaction, 40 fmoles of labeled probes was incubated with increasing amounts of crude extract or recombinant NMB1843 in 25 µl final volume of Gelshift Binding Buffer (25 mM Tris-HCl pH 7.5, 1mM $MgCl_2$, 10% glycerol) with 2µg salmon sperm DNA as non-specific competitor, for 15 min at room temperature, and run on 6% native polyacrilamide gels buffered with 0.5x TBE at 100 Volts for 80 min at 4 °C. Gels were dried and exposed to autoradiographic films at –80°C and radioactivity was quantified using a phosphorimager and the Image Quant software (Molecular Dynamics).

1.32 RNA preparation and Primer extension

N. meningitidis or *E. coli* strains were grown in liquid culture to logarithmic or stationary phase and then 20 ml of cultures were added to an equal volume of equivalent frozen

medium to bring the temperature immediately to 4 °C and then centrifuged at 3,000 rpm in a benchtop centrifuge at 4º C. RNA was extracted from the pelleted cells as previously described (Spohn, Beier et al. 1997). Primer extension was performed as previously reported (Delany, Ieva et al. 2003). To ensure correct mapping of the promoter sequencing reaction was carried out with a T7 sequencing kit (USB Corporation) using the same primer as in the primer extension reactions and the plasmid consisting of the relevant cloned promoter. Quantification of the signals from the primer elongated product was performed using Phosphoimager and ImageQuant software (Molecular dynamics). For quantitative experiments, experiments were performed from at least 2 independent biological replicates and figures show results from one representative experiment. Internal negative controls were performed on each RNA set quantifying the specific transcript of a gene whose expression is not altered, usually *adk* or nmb1870.

1.33 DNase I footprinting (NMB1843, *Neisseria gonorrhoeae* IHF, *Escherichia coli* RNApol and Alpha-sub)

The *nadA* promoter region was amplified from genomic DNA from different clinical isolates with the appropriate number of repeats as major clone and from pGFP-PΔ plasmid for 0 repeat, using primers nad-N1 and nad-B1 and cloned as 320, 342, 346 and 354 bp (for 0, 6, 7, 9 repeats respectively) *Nsi*I-*Bam*HI fragments into pGemT (Promega). A radioactive probe for DNA footprinting of *nadA* promoters were prepared as follows: approximately 2 pmol of the different plasmids were linearized with *Bam*HI, subjected to dephosphorylation using CIP (New England Biolabs), and then 5' end labelled using 5 pmol of [γ-32P]-ATP with T4 polynucleotide Kinase (New England

Biolabs). The plasmid was then digested with *Nsi*I, and *nadA* promoter fragments, labelled at one extremity, were purified by preparative polyacrylamide gel electrophoresis (PAGE). Briefly, the probe was extracted from a 6% polyacrylamide gel and eluted in 3 ml of elution buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 300 mM Na acetate [pH 5.2], 0.2% sodium dodecyl sulfate [SDS]) at 37°C overnight. The eluted probe was then extracted once with an equal volume of phenol-chloroform (1:1) and ethanol precipitated. Protein-DNA complexes were formed in 50 µl of footprinting buffer (20 mM Tris-HCl, pH 7.9, 50 mM KCl, 10 mM MgCl2, 0.01% NP-40, 10% glycerol) containing approximately 20 fmol (10 000 c.p.m) of the labelled probe and 200 ng of sonicated salmon sperm DNA as the non-specific competitor and recombinant NMB1843 protein, *Neisseria gonorreae* purified IHF (kindly donated by Hill SA, Belland RJ et al. 1997), *E. coli* RNA polymerase holoenzyme (USB) or alpha subunit (Tang, Murakami et al. 1996) in final concentrations as indicated, for 15 min at room temperature. Following incubation for 1 min at room temperature with 0.03 U of DNase I (Roche) and 5 mM $CaCl_2$, the reaction was stopped by addition of 140 µl of stop buffer (192 mM Na acetate, 32 mM Na2EDTA, 0.14% SDS, 64 mg ml-1 of sonicated salmon sperm DNA). Samples were phenol-extracted, ethanol-precipitated, re-suspended in denaturing sample buffer and fractionated on urea-6% polyacrylamide gels. As a molecular weight marker, a G+A sequence reaction (Maxam and Gilbert 1977) was performed for each DNA probe and run in parallel to the corresponding footprinting reactions.

1.34 MALDI TOF mass spectrometry

Proteins eluted from Dynabeads M-280 streptavidin column and dialyzed against H_2O were dried with a Speed Vac. (Labconco) and solubilized with 20 µl of 5 mM ammonium bicarbonate containing 0.1 % (wt/vol) of RapiGest™ SF surfactant

(Waters), incubated 5 min at 95°C and digested with 2 µg of trypsin (Sequencing grade Promega). The reaction was allowed to proceed for 15 hours at 37°C. An aliquot of the reaction was diluted 10 times with 0,1% (vol/vol) of trifluoroacetic acid, and 0.7 µl was directly spotted on a matrix PAC target (Prespotted AnchorChip 96, set for Proteomics, Bruker Daltonics). Air-dried spot was washed with 0.6 µl of a solution of 70% (vol/vol) ethanol, 0.1% (vol/vol) TFA. Peptide mass fingerprint spectra were recorded with a MALDI-TOF/TOF mass spectrometer UltraFlex (Bruker Daltonics). Ions generated by laser desorption at 337 nm (N_2 laser) were recorded at an acceleration of 25 kV in the reflector mode. In general, about 200 single spectra were accumulated for improving the signal/noise ratio and analyzed by FlexAnalysis (version 2.4, Bruker Daltonics). External calibration was performed using standard peptides pre-spotted on the target. The data of MS were further analyzed through an in-house licensed MASCOT, version 2.2.1 (Matrixscience Ltd), running on a local server containing the protein sequence data downloaded from NCBI. The following parameters were used for database searches: monoisotopic mass accuracy, 75 pm, missed cleavages, 1, oxidation of methionine as variable modifications.

Table 1. Oligonucleotides used in this study

Name	**Sequence**	**Site**
Nad-N1	attcagatgcatGACGTCGACGTCCTCGATTACGAAGGC	NsiI
Nad-N2	attcagatgcatTAAGACACGACACCGGCAGAATTG	NsiI
Nad-N3	attcagatgcatCCGAACTACCTAACTGCAAG	NsiI
Nad-N4	attcagatgcatTTGCGACAATGTATTGTATATATG	NsiI
Nad-N5	attcagatgcatCTTTAATATGTAAACAAACTTGGTGG	NsiI
Nad-Sp	attcagcatgctacGCTCATTACCTTTGTGAGTGG	SphI
Nad-B1	attcaggatcctacGCTCATTACCTTTGTGAGTGG	BamHI
n-85/50F	CTACCTAACTGCAAGAATTcTTGCGACAATGTATTG	EcoRI
n-85/50R	CAATACATTGTCGCAAgAATTCTTGCAGTTAGGTAG	EcoRI
Bio-nad-N2	attcagatgcatTAAGACACGACACCGGCAGAATTG	NsiI
nad-Aa2	attcaggacgtcTAAGACACGACACCGGCAGAATTG	AatII
nad-Nh	attcagctagcCATGCTCATTACCTTTGTGAGTGG	NheI
1843-1	TACGTTCCGGCAGTTCGGCGG	
1843-2b	cgcatcctcgggatccGGGTAGGCATTGTTTAAGTCTCC	BamHI
1843-3b	caaatgcctacccggatccCGAGGATGCGTTGAACTCGTAATACGCCG	BamHI
1843-4	ACCGCTCTTCGGGCGACAGGCCGG	
1843-F	attcacatATGCCTACCCAATCAAAACATGCG	NdeI
1843-R	attcaggatcCGGCGTATTACGAGTTCAACGCATCCTCG	BamHI
IHF-Lex	ctagaACTGCAAGAATTAAATAAATAAATAAATAAATAAATTGCGAC	XbaI
IHF-Rex2	ctagaGTCGCAATTTATTTATTTATTTATTTATTTAATTCTTGCAGT	XbaI

Gpe-3	GAATTGGGACAACTCCAGTG	
1870-pe	gaatcagggcagtggtcagag	
Adk-PE	CGCGCCTAAAAGTAATGC	
gpr-R	gattagcatgcCGGCATTAATATCTGTTAATATGTGC	SphI
SR-F	TCGGAAGCCGTCCGTTCCGAACC	
SR-R	attatggatccATAAACGCCAAACCCACCGCGAAGGTGG	BamHI

[a] Capital letters indicate *N. meningitidis* derived sequences, small letters indicate sequences added for cloning purposes, and underlined letters indicate restriction enzyme recognition sites.

Table 2. Strains and Plasmids used in this study.

Name	Relevant characteristics	Reference or source
Neisseria meningitidis		
MC58	Clinical isolate, sequenced strain containing 9 TAAA tetranucleotide repeats in the *nadA* promoter	(Tettelin, Saunders et al. 2000)
5/99	Clinical isolate containing 8 TAAA tetranucleotide repeats in the *nadA* promoter	Norwegian clinical isolate
BZ83	Clinical isolate containing 5 TAAA tetranucleotide repeats in the *nadA* promoter	(Comanducci, Bambini et al. 2002)
ISS838	Clinical isolate containing 6 TAAA tetranucleotide repeats in the *nadA* promoter	(Comanducci, Bambini et al. 2002)
961-5945	Clinical isolate containing 12 TAAA tetranucleotide repeats in the *nadA* promoter	(Comanducci, Bambini et al. 2002)
MC-Δ1843	1843 null mutant in MC58 strain, Cm^R	This study
5/99-Δ1843	1843 null mutant in 5/99 strain, Cm^R	This study

BZ83-Δ1843	1843 null mutant in BZ83 strain, Cm^R	This study
ISS-Δ1843	1843 null mutant in ISS838 strain, Cm^R	This study
961-Δ1843	1843 null mutant in 961-5945 strain, Cm^R	This study
MC-P(1-5)	Series of 5 derivatives of MC58, containing single copy transcriptional fusion of 5' deletions of the *nadA* promoter fused to the *gfp* gene, Ery^R	This study
MC-PΔ	Derivative of MC58, containing single copy transcriptional fusion of a mutant variant of the *nadA* promoter, with the tetranucleotide repeats deleted, fused to the *gfp* gene, Ery^R	This study
MC-P2(x4-x13)	Series of 10 derivatives of MC58, containing single copy transcriptional fusion of the *nadA* P2 promoter variants, containing from 4 to 13 tetranucleotide repeats, fused to the *gfp* gene, Ery^R	This study
Δ1843-PΔ, -P2(x6, x8, x9)	4 Derivatives of MC-Δ1843, containing single copy transcriptional fusion of the *nadA* P2 promoter with either 0, 6, 8 or 9 tetra nucleotide repeats, fused to the *gfp* gene, Ery^R	This study
MC-Fko	Fur null mutant of MC58	(Delany, Ieva et al. 2003)
Fko-P2(x9)	Derivative of MC-Fko containing single copy transcriptional fusion of the *nadA* P2 promoter with 9 repeats, fused to the *gfp* gene, Ery^R	This study
Plasmids		
pGEMT	Cloning vector, Amp^R	Promega
pΔ1843ko::Cm	Construct for generating knockout of the NMB1843 gene, Cm^R	This study
pGFP	Construct for insertion of *nadA* promoter variants and mutants fused to *gfp* in single copy between ORF nmb1074 and NMB1075 in the *N. meningitidis* chromosome, AmpR, Ery^R	(Ieva, Roncarati et al. 2008)
pGX-1	Derivative pSC101 containing the *gfp* gene, CmR	(Urban and Vogel 2007)

pGX-nad	Derivative of pGX-1 (Urban et al. 2007) with the *nadA* promoters cloned as a AatII/NheI fragment upstream of the *gfp* gene, Cm^R	This study
plaw2	Expression vector for over-expression of alpha subunit of RNA polymerase under an IPTG-inducible promoter, Amp^R	(Zou, Fujita et al. 1992)
plaw2Δ(1-235)	Expression vector for over-expression of N-terminus of the alpha subunit of RNA polymerase from amino acids 1-235, under an IPTG-inducible promoter, Amp^R	(Zou, Fujita et al. 1992)
pHTT7f1-NHα	Vector for expression of N-terminal Histagged alpha subunit of RNA polymerase protein under an IPTG-inducible promoter, Amp^R	(Tang, Severinov et al. 1995)
pET15b	Expression vector for N-terminal Histagged proteins, Amp^R	Invitrogen
pET15b-1843	pET15b derivative for expression of recombinant 1843 protein, Amp^R	This study

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