Regulation of class II pilin gene expression in *Neisseria meningitidis*

Mariya Lobanovska

Sir William Dunn School of Pathology
St Anne’s College
University of Oxford

A thesis submitted in partial fulfilment of the requirements for the degree of
Doctor of Philosophy
Trinity Term 2018

Regulation of class II pilin gene expression in *N. meningitidis*

Mariya Lobanovska, DPhil Pathology, Trinity Term 2018, St Anne's College

Abstract

Neisseria meningitidis expresses multi-component organelles called Type four pili (Tfp), which are key virulence factors involved in interactions with host cells. Pilin (PilE) is the main component of Tfp and *N. meningitidis* can express either class I pilins that undergo antigenic variation or class II pilins, which are highly conserved. The region upstream of the *pilE* gene contains two putative promoters which are recognised by distinct σ (sigma) factors: σ^{70} family and σ^{N}. We aimed to investigate regulation of class II *pilE* by characterising the role of multiple σ factors in class II *pilE* transcription.

We confirmed the presence of conserved σ^{70} family- and σ^{N}-dependent promoter sequences in a large collection of meningococcal genomes. We show that the promoters are functional in *E. coli*, however in *N. meningitidis* PilE expression under steady state is initiated from the σ^{70} family-dependent promoter. Furthermore, neither the absence nor overexpression of σ^{N} had any effect on pilin levels in *N. meningitidis* which indicates that σ^{N} does not contribute to class II pilin regulation, and also suggests that σ^{N} does not function as a -12/-24 sequence-dependent activator. Strikingly, *Neisseria elongata* σ^{N}, which is known to function as an activator of *pilE* in commensal *N. elongata*, was able to reduce meningococcal pilin expression. The negative effect exerted by *N. elongata* σ^{N} on class II pilin expression raises a possibility that in pathogenic *Neisseria* the σ-dependent network responsible for the expression of *pilE* has been modified to maintain Tfp expression, considering their key role in colonisation and pathogenesis.

Studying transcription patterns of virulence factors offers an interesting possibility to understand features that define pathogenic *Neisseria*. We analysed σ^{H} regulon in *N. meningitidis* by RNA sequencing and our results suggest that σ^{H} might be implicated in the response to diverse stimuli including the canonical heat shock response.

In summary, results presented here provide new insights into class II *pilE* regulation and the role of σ^{H} in *N. meningitidis*. Future work will focus on identifying signalling networks that govern *pilE* expression in response to environmental stimuli and will explore the σ factor cross-talk in pathogenic *Neisseria*. This will expand our knowledge of meningococcal adaptation to niches within the host.

Acknowledgements

I would like to thank my supervisors Professor Christoph Tang and Dr Rachel Exley for their time, patience and for giving me so many wonderful opportunities. I am really grateful for your support, encouragement and advice.

I would like to express my gratitude to my examiners Professor Peter Cook and Dr Mark Thomas for their comments and helpful suggestions. I want to thank Dr Aartjan te Velthuis for advice on Primer extension analysis and Dr Vladimir Pelicic for *Neisseria meningitidis* genomic DNA. I thank Vertis Biotechnologies for cDNA library preparation, Illumina sequencing and initial bioinformatic analysis of RNA-sequencing data.

I would also like to thank everyone in the Tang lab for making my PhD such an amazing experience. During my four years I had a chance to work alongside some incredible people who I have become really close friends with and I will cherish fond memories of my time in the Tang lab.

I would like to thank the Foundation for supporting my studies and the Dunn School for providing an outstanding research environment and a great place to work.

Finally, thank you to my parents and my family for their love and for always helping me.

Abbreviations

Abbreviation	Full name
bp	Base pair
DNA	Deoxyribonucleic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
nt	nucleotide
OD600	Optical density measured at 600 nm
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
σ factor	Sigma factor
SSC	Saline sodium citrate
SDS	Sodium dodecyl sulfate
TFP	Type four pili

Table of Contents

List of Figures

List of Tables

1 Introduction

1.1 *Neisseria meningitidis*

1.1.1 *Neisseria* genus

N. meningitidis is a member of the *Neisseria* genus that includes two pathogenic species, *N. meningitidis* and *Neisseria gonorrhoeae*, as well as non-pathogenic or commensal *Neisseria* which have been isolated from humans and animals (Liu *et al.*, 2015). *Neisseria meningitidis* is a human-specific Gram-negative diplococcus that is a leading cause of meningitis and septicaemia worldwide (Diallo *et al.*, 2016; Stephens *et al.*, 2007). Despite its ability to cause invasive disease, *N. meningitidis* is a commensal that is carried asymptomatically by approximately 10% of the population (Trotter *et al.*, 2005). *N. meningitidis* occupies the mucosal niche of the human nasopharynx (Rosenstein *et al.*, 2001). However, some meningococcal isolates have been isolated from the urogenital tract (Harrison *et al.*, 2017). *N. gonorrhoeae* is the causative agent of the sexually transmitted disease gonorrhoea. *N. gonorrhoeae* preferentially colonises the urogenital epithelium but some gonococcal strains have been isolated from nasopharyngeal and mucosal niches (Danby *et al.*, 2016; Noble *et al.*, 1979).

Commensal *Neisseria* contains a diverse group of species however, our knowledge of their biology is limited. More than eight commensal *Neisseria* are considered part of the human nasopharyngeal flora (Zaura *et al.*, 2009) and although generally considered non-pathogenic, some commensal *Neisseria* have been reported to cause the disease in immunocompromised patients (Zavascki *et al.*, 2006). The most well studied member of the commensal species is

N. lactamica (Marri *et al.*, 2010). Studies have demonstrated that the rate of *N. lactamica* carriage decreases with age whereas *N. meningitidis* colonisation starts in children and increases until it reaches its peak in young adults, suggesting that *N. lactamica* can induce protective immunity against *N. meningitidis* (Bennett *et al.*, 2005; Evans *et al.*, 2011). Another member of the commensal flora of the nasopharynx is *N. elongata*, which in rare cases has been reported to cause endocarditis (Nawaz *et al.*, 1996). It is the most distantly related species to *N. gonorrhoeae* and interestingly the two species have been shown to interact and co-inhabit the same pharyngeal niche (Higashi *et al.*, 2011).

1.1.2 Classification of *N. meningitidis*

N. meningitidis possesses a polysaccharide capsule which plays an important role in bacterial evasion from host immune responses and in transmission (Spinosa *et al.*, 2007; Swartley *et al.*, 1997). The presence of the capsule is critical for bacterial spread and survival in the bloodstream as capsule offers protection from antibody-driven adaptive immunity and complement-induced lysis. The meningococcal capsular polysaccharide forms the basis of meningococcal classification into serogroups. There are 12 serogroups of *N. meningitidis* but the majority of disease is caused by serogroups A, B, C, W, X and Y (Harrison *et al.*, 2013). The capsule of B, C, Y and W groups consists of polysialic acid linked to glucose or galactose whereas group A expresses *N*-acetyl mannosamine-1-phosphate containing capsules (Liu *et al.*, 1971). The α2-8 linkage of polysialic acid in group B capsule is similar to the polysialic acid on the host mammalian neural cell adhesion molecule (NCAM) which impairs host immune response against serogroup B strains (Finne *et al.*, 1987). *N. meningitidis* is a naturally transformable organism and its genome is relatively small ~2.2 Mb (Tettelin *et al.*, 2000). The genome contains a number of different repetitive sequences, which are scattered throughout the chromosome and can contribute to multiple recombination events (Liu *et al.*, 2002). The ability to recombine in part explains the ability of meningococcus to acquire new genetic determinants, including variation in capsule encoding genes (Schoen *et al.*, 2009). Recombination events can lead to the exchange of the regions within the capsule loci between different serogroups, which results in capsule switching (Swartley *et al.*, 1997).

The development of the multilocus sequence typing (MLST) tool in 1998 allowed further classification of *N. meningitidis* (Maiden *et al.*, 2013). MLST relies on the analysis of seven housekeeping genes. When a new sequence variant at a specific locus is identified, it is assigned an arbitrary allele number, and the pattern of allele numbers determines the sequence type (ST). Strains with similar STs, such as those that match at four or more loci, are grouped into clonal complexes (cc) (Caugant, 2008). Comparison of *N. meningitidis* isolates has led to the observation that *N. meningitidis* exists in clusters of clones that share enough genetic determinants to identify the common ancestor. Studies examining disease isolates and the biology of meningococcal populations have identified a subset of hyperinvasive lineages of *N. meningitidis* which included cc 5, cc 11, cc 269, cc 32 and cc 41/44 (Caugant *et al.*, 2009). These hyperinvasive lineages are only occasionally present in carriers, and are associated mostly with disease. Importantly, understanding the lineages of *N. meningitidis* helps to identify the spread and global evolution of meningococcus.

1.1.3 Meningococcal disease manifestation and epidemiology

N. meningitidis is transmitted *via* respiratory droplets upon close contact. Meningococcal disease has significant implications for public health as onset is very rapid and has a 10%

fatality rate (Rosenstein *et al.*, 2001). Invasive meningococcal disease results in systemic infection, often leading to septic shock, with a 40% mortality rate in these cases (Chang *et al.*, 2012). Even when antibiotic treatment is administered early, patients often develop disease sequalae including cognitive impairment, limb loss and deafness (Borg *et al.*, 2009). Host susceptibility factors including absence of bactericidal antibodies and genetic impairments in innate immune defence pathways play an important role in development of disease (Lewis *et al.*, 2014). Furthermore, environmental factors and age further contribute to susceptibility to meningococcal disease (Lewis & Ram, 2014). Meningococcal carriage and disease rate are elevated in closed populations, for instance among military recruits or university students. Furthermore, factors such as co-infections (*e.g.* influenza), smoking and damage to the mucosal surface also increase meningococcal disease incidence (Stephens *et al.*, 2007). Progression and severity of meningococcal sepsis depend on the survival and presence of high number of bacteria in the blood. Bacterial invasion is followed by aberrant inflammation and host tissue damage. Endotoxin (lipopolysaccharide, LPS) released by *N. meningitidis via* outer membrane vesicles is the main trigger of host inflammatory response (Brandtzaeg, Kierulf*, et al.*, 1989). LPS is able to bind endotoxin binding protein present in the human plasma, which leads to activation of inflammatory cells including macrophages and neutrophils. LPS-activated macrophages trigger the release of proinflammatory cytokines (TNF-α, IL-1β) and neutrophils undergo respiratory burst and degranulation, releasing reactive oxygen species and inflammatory proteins. Together this causes acute inflammation and meningococcal septicemia, which impairs microvasculature and leads to multi-organ failure (Pathan *et al.*, 2003). During meningococcal sepsis vascular permeability increases which leads to respiratory failure and myocardial dysfunction. Furthermore, thromboresistance and intravascular coagulation are impaired resulting in the characteristic purpura fulminans (Lécuyer *et al.*, 2017).

Historically, *N. meningitidis* has caused a number of epidemics worldwide. A serogroup A outbreak in a region of sub-Saharan Africa known as the meningitis belt has resulted in periodic epidemics over the last 70 years (Harrison *et al.*, 2009), and the highest incidence of the disease cases ever recorded (LaForce *et al.*, 2009). Serogroup C epidemics have also been observed in Niger and more recently, in 2002, a serogroup W outbreak resulted in epidemics in Burkina Faso (Nathan *et al.*, 2007). Compared to African countries, the overall disease rate is low in Europe and the USA. However occasional epidemics of serogroup B and C are observed.

Vaccination remains one of the most effective ways to prevent meningococcal disease and in some cases can reduce the carriage state. Introduction of a trivalent A/C/W vaccine lead to near disappearance of W strains in Burkina Faso (Traore *et al.*, 2006). Meningococcal polysaccharide A/C vaccine was used to control meningococcal outbreaks, however poor immunogenicity in infants and absence of herd immunity limit the affinity of these vaccines to offer full protection in countries experiencing serogroup A epidemic. Subsequent development of a conjugate meningococcal A vaccine in 2010 lead to a 99% reduction in serogroup A disease in the vaccinated population in nine countries belonging to meningitis belt (Trotter *et al.*, 2017). Currently there are a number of vaccines available, including a quadrivalent ACWY conjugate that is protective against all *N. meningitidis* strains present predominantly in sub-Saharan Africa; and conjugate MenC vaccine which was introduced into routine vaccination in the UK in 1999 and lead to a fall in Serogroup C incidence

(Campbell *et al.*, 2009); as well as a conjugate MenB vaccine which has recently been introduced in the UK and is now part of routine immunisation schedule (Giuliani *et al.*, 2006)

1.2 Host adaptation in *N. meningitidis*

N. meningitidis colonisation can be transient or can persist for up to several months (Christensen *et al.*, 2010). Establishing successful colonisation in the nasopharynx is critical for bacterial survival in the host. Once colonized, *N. meningitidis* can occasionally translocate across the epithelial barrier and enter the bloodstream causing systemic meningococcal disease (Hill *et al.*, 2010). The bacteria can then spread and progress further to cross blood brain barrier, and enter cerebrospinal fluid causing fulminant meningitis. Interestingly, progression to invasive disease does not provide a fitness advantage to *N. meningitidis* as it impairs its transmission from host to host. Therefore, it is likely that the systemic disease is a result of an accidental event as *N. meningitidis* is largely an asymptomatic colonizer (Buckee *et al.*, 2008).

To meet the challenges of the distinct conditions encountered during the colonisation and disease, *N. meningitidis* possesses two crucial determinants: 1) virulence factors and 2) metabolic attributes which include strategies to utilize host resources for replication.

Virulence factors include adhesins, capsule, LPS, and other surface molecules involved in host immune evasion. Some of the best characterised virulence determinants are described below.

(i) Adhesins expressed by *N. meningitidis* are required depending on the stage of attachment, and can be divided into major and minor adhesins (Hill *et al.*, 2010). Initial attachment to cells is mediated by the major adhesin Type four pili (Tfp) and it has been suggested that Tfp bind CD46 on host cells (Kallstrom *et al.*, 1997). However there are conflicting reports describing Tfp-dependent adhesion in the absence of CD46 (Kirchner *et al.*, 2005; Tobiason *et al.*, 2001). Tfp take part in attachment and microcolony formation on the surface of epithelial cells in the nasopharynx and on endothelial cells of the vasculature. However the signaling triggered by Tfp-mediated adhesion differs between the two cell types (Lecuyer *et al.*, 2012). For example, when *N. meningitidis* attaches to endothelial cells, the β-2 adrenergic receptor/ β-arrestin pathway is activated which triggers a signaling cascade, allowing bacterial to cross cell monolayers (Coureuil *et al.*, 2010). Attachment of bacteria to epithelial cells does not engage of any of these pathways, suggesting the downstream Tfp-induced intracellular signaling is different in endothelial and epithelial cells.

Other major adhesins include outer membrane opacity proteins (Opa and Opc) which have been shown to bind a number of different carcinoembryonic antigen cell adhesion molecules (CEACAM), and are implicated in mediating prolonged adhesion with host epithelial cells (Virji *et al.*, 1996). Minor adhesins, including NhhA, App and NadA, enable engagement of a diverse range of receptors and are proposed to be recruited for adhesion at different stages of pathogenesis (Pizza *et al.*, 2015).

(ii) Capsule and LPS are important for meningococcal immune evasion. Capsule expression is mediated by a number of genes in the capsule encoding locus (*cps*) (Frosch *et al.*, 1989). The *Cps* locus is composed of regions A, B and C responsible for production, modification and transport of the capsule components respectively. Meningococci have developed strategies to vary the presence of the capsule on the surface (Hammerschmidt *et al.*, 1996) and downregulation of the capsule has been shown to enhance the initial adhesion to host cells during disease (Deghmane *et al.*, 2002). Furthermore, capsule is critical for meningococcal survival in the bloodstream in the host (Jarvis *et al.*, 1987). LPS present in the outer cell membrane comprises of a conserved membrane-associated lipid A component, which anchors variable α- and β- chains (Kahler *et al.*, 1998; Kulshin *et al.*, 1992). Lipid A of LPS triggers the aberrant inflammatory response, that characterises meningococcal disease, by activating Toll-like receptor 4 (TLR4) (van Deuren *et al.*, 2000), and the concentration of LPS in human blood correlates with severity of the disease (Brandtzaeg, Mollnes*, et al.*, 1989; Fransen *et al.*, 2010).

(iii) Exotoxins. In general exotoxins can affect neighbouring bacteria as well as human cells and have been described in many organisms, however our knowledge of toxins secreted by pathogenic *Neisseria* is limited. *N. meningitidis* has been reported to contain a polymorphic toxin named Multiple Adhesin Family protein B (MafB), which is thought to provide a fitness advantage to bacteria in polymicrobial environments and can degrade both bacterial and human RNA (Jamet *et al.*, 2015). Gene encoding MafB is located on a pathogenicity island flanked by insertion sequences, which are thought to facilitate the acquisition of these islands *via* horizontal gene transfer (Jamet *et al.*, 2015).

(iv) A number of surface molecules contribute to meningococcal virulence. These include factor H binding protein, which recruits a negative complement regulator factor H to escape recognition and killing by complement (Schneider *et al.*, 2009). In addition, *N. meningitidis* possesses membrane channels known as porins (PorA and PorB), which have been implicated in both colonisation and immune evasion. For example, PorA has been shown to interact with complement inhibitor C4bp to increase bacterial survival in the blood (Jarva *et al.*, 2005).

Metabolic attributes allow *N. meningitidis* to acquire nutrients to regulate metabolism in order to exploit the resources available in the various niches it encounters. For example, iron acquisition is essential for *N. meningitidis* survival (Archibald *et al.*, 1978) and *N. meningitidis* expresses multiple iron uptake systems, which are used to retrieve iron from human iron-binding proteins (Perkins-Balding *et al.*, 2004). It has been proposed that different host iron-binding proteins such as transferrin and haemoglobin serve as niche indicators for *N. meningitidis* by activating distinct transcriptional responses that contribute towards bacterial survival (Jordan *et al.*, 2009). Many metabolically linked genes have been implicated in meningococcal colonisation. Five meningococcal genes have recently been identified belonging to metabolic pathways that play a crucial role in bacterial attachment to epithelial cells (Jamet *et al.*, 2013). In line with this observation, deletion of the gene encoding a lactate permease (*lctP*) leads to a decrease in bacterial survival when incubated with explants from nasopharyngeal mucosa (Exley *et al.*, 2005). Interestingly, lactate catabolism has also been implicated in progression of invasive disease. By utilizing lactate as a carbon source, *N. meningitidis* enhances the biosynthesis of sialic acid that is incorporated on its surface. Increased presence of sialic acid inhibits binding of the complement factor C3 to the bacterial surface, thus preventing complement-mediated killing (Exley *et al.*, 2005).

1.3 Type four pili (Tfp)

Tfp are long filamentous structures present on the surface of *N. meningitidis* (Kolappan *et al.*, 2016). Among meningococcal factors that allow the bacteria to colonise the nasopharynx, Tfp play a key role by enabling bacteria to form microcolonies on all surfaces (Nassif *et al.*, 1993; Virji *et al.*, 1992). Tfp are multicomponent dynamic filaments that span the inner and outer membrane of *N. meningitidis*. The absence of Tfp components significantly reduces the ability of bacteria to attach to host cells (Nassif *et al.*, 1994; Virji *et al.*, 1992). Meningococcal dissemination within the host is proposed to be triggered by the detachment of a small number of bacteria from microcolonies on the epithelial surface, and translocation of these bacteria across the epithelial layer to the systemic circulation (Melican *et al.*, 2012). Subsequent Tfp-mediated formation of microcolonies on endothelial cells provides resistance against shear stress in the circulation (Coureuil *et al.*, 2009). In the brain microvasculature, the local reorganisation of the host cell cytoskeleton, which is triggered by Tfp binding to the cells, promotes translocation of *N. meningitidis via* the paracellular route and bacterial spread to the meninges (Mikaty *et al.*, 2009). In addition, Tfp mediate twitching motility and the uptake of DNA that enables horizontal gene transfer (Cehovin *et al.*, 2013; Eriksson *et al.*, 2015)

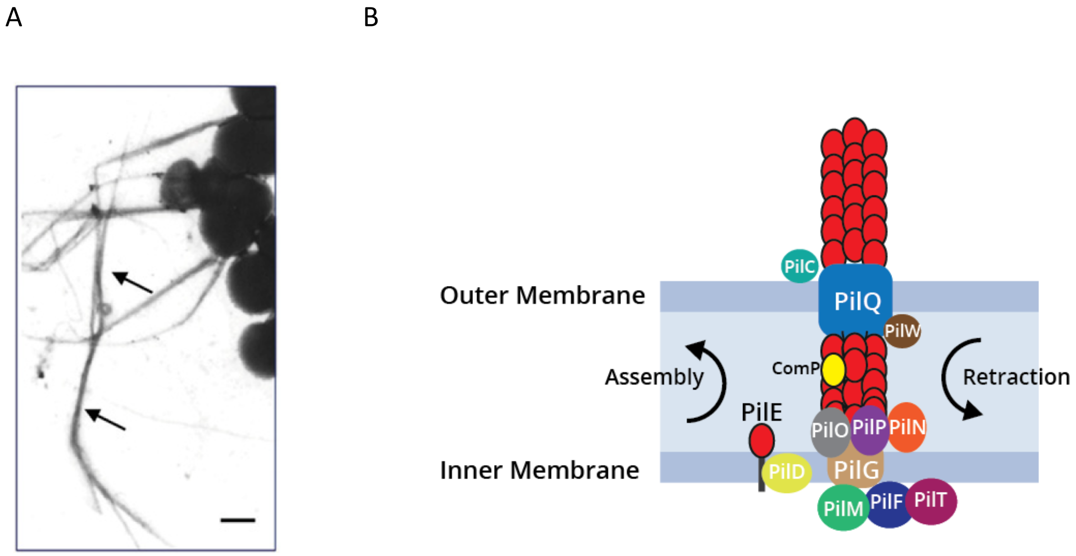


Figure 1.1 Type four *pili* of *N. meningitidis*

A. Arrows showing Type four pili fibres on the surface of *N. meningitidis* (transmission electron micrograph, 0.5 µM; from (Hill *et al.*, 2010). B. Schematic diagram of components of Tfp biogenesis apparatus.

Tfp are encoded by over 15 biogenesis genes that are distributed around the meningococcal genome (Brown *et al.*, 2010; Kolappan *et al.*, 2016). The main component of the fibre is the pilin protein which is encoded by *pilE* gene (Figure 1.1B). PilE is first made as a prepilin which is targeted to the inner membrane. Then prepilin is cleaved by the PilD prepilin peptidase, leaving the mature PilE with a conserved N terminal sequence, which is critical for assembly, into a fibre. There are a number of proteins which are incorporated in the Tfp fibre that have a similar structure to PilE (Cehovin *et al.*, 2010), including ComP, PilX and PilV. ComP mediates DNA binding, providing DNA uptake sequence (DUS) is present in the incoming DNA. DUS are repetitive sequences in the meningococcal genome that facilitate DNA uptake and subsequent recombination thus increasing genetic diversity

(Treangen *et al.*, 2008). PilX is required for bacterial aggregate formation, and deletion of *pilX* reduces bacterial attachment to cells (Helaine *et al.*, 2005). PilV deletion alters the Tfp-dependent adhesion and signalling to host cells: *pilV* mutants do not form stable microcolonies and twitching motility is increased, suggesting that *pilV* is involved in microcolony stability (Brown *et al.*, 2010; Imhaus *et al.*, 2014).

Additional Tfp components positioned at the cytoplasmic side of the inner membrane include PilG which is essential for fibre formation, and two AAA+ ATPases: PilF and PilT (Figure 1.1B). PilF is required for Tfp expression and PilT is essential for Tfp retraction. The outer membrane contains PilQ, which is also required for Tfp biogenesis (Collins *et al.*, 2001). PilQ forms a pore-like structure composed of several subunits and this arrangement is maintained by additional proteins such as PilW, PilP and Omp85. PilC is associated with the pilus fibre and is encoded by *pilC1* and *pilC2*. PilC is phase variable, which allows further modification of Tfp function (Rytkönen *et al.*, 2004). Interestingly, only *pilC1* is crucial for *N. meningitidis* adhesion whereas both PilC1 and PilC2 modulate Tfp expression and DNA competence (Morand *et al.*, 2001).

1.4 PilE: the major subunit of Tfp

N. meningitidis expresses one of two classes of pilin, class I that undergoes antigenic variation, or invariant class II pilin (Aho *et al.*, 1997; Virji *et al.*, 1989). Interestingly the distribution of class I and class II *pilE* varies among isolates. Class I *pilE* is expressed in a diverse range of strains isolated from patients with disease and carriers whereas class II *pilE* is present almost exclusively in a subset of meningococcal hypervirulent lineages that are largely associated with epidemic disease. The two classes were originally identified based on their reactivity with a monoclonal antibody SM1 raised against *N. gonorrhoeae* pilin; SM1 exclusively recognises class I pilin (Virji *et al.*, 1989). Further analysis revealed distinct genomic organisation of genes encoding class I and class II pilin (Figure 1.2) (Aho *et al.*, 1997; Cehovin *et al.*, 2010; Wörmann *et al.*, 2014).

Less is known about the structure/function of Tfp in the commensal species; however, work on *N. cinerea* has shown that in contrast to pathogenic species, Tfp is not required for adhesion in these species, while in *N. elongata* pilin has been shown to play a key role in the ability of bacteria to take up DNA and interact with *N. gonorroheae* (Higashi *et al.*, 2011). There are two putative *pilE* sequences in the genomes of commensal *Neisseria*, *pilE1* and *pilE2* (Aho *et al.*, 2000). PilE1 and PilE2 exhibit sequence homology with class II *pilE* at both the N and C termini. *pilE1* and *pilE2* are arranged in tandem; however, in commensal *N. cinerea* only *pilE1* transcription is detectable under standard growth conditions (Wörmann *et al.*, 2016).

1.4.1 Antigenic variation of *pilE*

Class I *pilE* is located in close proximity to up to eight copies of *pilS* cassettes, which are homologous to *pilE* but are not expressed (Haas *et al.*, 1986). Antigenic variation (Av) is a consequence of the recombination of these *pilS* cassettes with *pilE*, which results in a change in *pilE* sequence. Occasionally such DNA recombination results in the generation of inactive *pilE* leading to abolished expression of PilE and phase variation (Cahoon *et al.*, 2011). A DNA recombination event at the *pilE* locus can also generate additional PilD-dependent cleavage sites within PilE. As a result, a truncated soluble pilin (S-pilin) is produced. S-pilin is unable to assemble into a functional fibre. Therefore, strains containing these variants of *pilE* lack Tfp and have impaired adhesion (Obergfell *et al.*, 2016). Overall pilin Av is proposed to serve as a mechanism to evade the immune recognition by circumventing pilin-directed humoral responses and has been shown to modulate adhesion (Miller *et al.*, 2014).

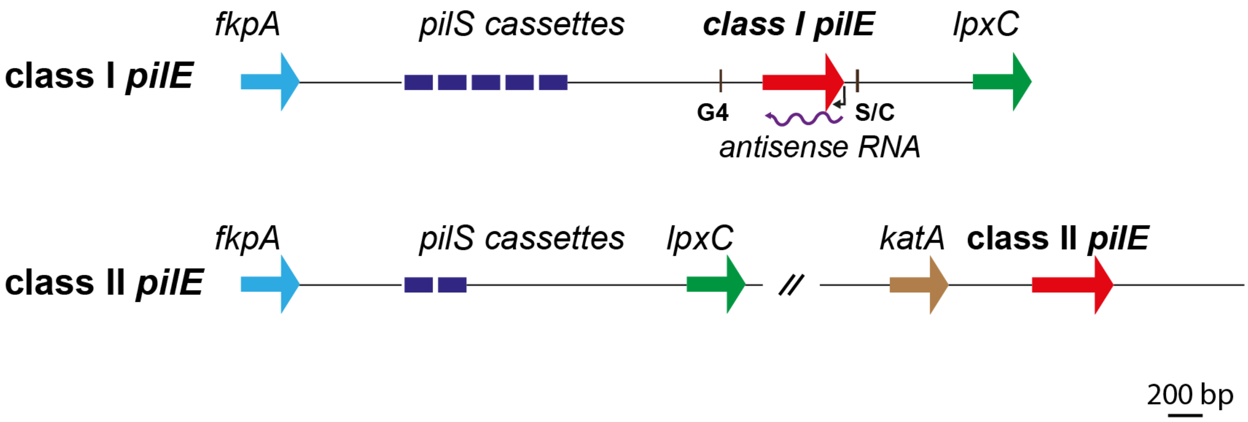


Figure 1.2 Diagram of loci encoding class I and class II pilin

Class I *pilE* is flanked by *fkpA* and *lpxC* genes. Several *pilS* cassettes and a G4 as well as Sma/Cla sequence (S/C; downstream of *pilE*) together contribute to pilin antigenic variation. The class II *pilE* gene is in a different location and lacks the G4 element and Sma/Cla sequence. *pilS* cassettes are present in class II *pilE*-expressing isolates but do not act as donors for homologous recombination and are not in the same locus as in class I *pilE*.

The mechanism behind Av has not been fully defined. However, the two elements, G4 and Sma/Cla present upstream and downstream of class I *pilE* respectively, are necessary for pilin Av (Figure 1.2). G4 is a stretch of guanine rich sequence which functions as a recombination initiation sequence structure and mutagenesis of G4 inhibits *pilE* Av (Cahoon *et al.*, 2009, 2013). The Sma/Cla repeat is a homologue of a recombinase binding sequence and is only present in pathogenic *Neisseria*. Sma/Cla has been shown to facilitate Av in *N. gonorrhoeae*. However, the underlying mechanism by which Sma/Cla attributes to Av is not fully understood (Wainwright *et al.*, 1997).

pilE Av is also regulated by small regulatory RNAs. In addition to noncoding RNA that is initiated from the promoter that overlaps with the G4 sequence (Cahoon & Seifert, 2013), antisense RNA is transcribed from the promoter situated at the 3' end of class I *pilE* in 8013 and modulates the frequency of *pilE* Av (Figure 1.2) (F. Y. Y. Tan *et al.*, 2015).

In contrast to class I *pilE*, class II *pilE* is located at a different chromosomal locus, and despite containing 1-2 *pilS* cassettes elsewhere on the genome, it does not undergo Av (Helm *et al.*, 2010). Importantly, the class II *pilE* locus lacks both G4 and Sma/Cla repeat sequence which could account for the lack of Av (Aho *et al.*, 2000; Wörmann *et al.*, 2014).

1.4.2 Regulation of pilin expression in *N. meningitidis*

Pilin expression is controlled at multiple levels including transcriptional, post-transcriptional and post-translational (Figure 1.3).

Transcriptional. At the genomic level, class I and class II *pilE* have different promoter organisations. Both class I and class II *pilE* contain two putative promoters -10/-35 and -12/-24 sequences, which are annotated based on the known bacterial promoter consensus sequences. However the sequence and the position of these promoter regions relative to the *pilE* start codon is distinct between the two classes. This will be discussed further in Chapter 3. In *N. cinerea* and *N. elongata pilE1* and *pilE2* contain putative -10/-35 promoter regions and *pilE1* harbours an additional -12/-24-dependent promoter, which in *N. elongata* has been shown to activate transcription of *pilE* (Rendon *et al.*, 2013; Wörmann *et al.*, 2016). The regulation of class II *pilE* expression has not been addressed before. Understanding the regulation of class II *pilE* gene transcription in *N. meningitidis* forms the subject of this thesis.

Class I *pilE* is known to be transcribed from the -10/-35 promoter (Carrick *et al.*, 1997) and several transcription factors have been implicated in class I *pilE* regulation, including contact regulatory gene A (CrgA). The role of CrgA in *pilE* regulation is strain-dependent (Deghmane *et al.*, 2000; Ieva *et al.*, 2005). In *N. meningitidis* 8013 which contains class I *pilE*, CrgA is thought to downregulate *pilE* via its interaction with a CrgA-binding site upstream of *pilE* (Deghmane *et al.*, 2002). In *N. meningitidis* MC58, which also expresses class I *pilE*, CrgA has been similarly shown to bind the *pilE* promoter *in vitro* (Derkaoui *et al.*, 2016). However, CrgA-dependent pilin regulation was not observed in this strain (Ieva *et al.*, 2005).

Furthermore, stress signals have been shown to affect *pilE* expression in *N. gonorrhoeae*, which expresses a *pilE* gene homologous to meningococcal class I *pilE* (Larribe *et al.*, 1997). Gonococcal pilin expression is affected by various environmental conditions including low iron, high osmolarity and pH; and this modulation is thought to be governed by a two-protein component system which consists of a response-regulator PilA that binds *pilE* promoter and putative sensor/histidine kinase component PilB (Larribe *et al.*, 1997).

Post-transcriptional. *Neisseria* anti-aggregation protein (NafA) has been suggested to regulate pilin protein but not *pilE* transcript levels in a class II *pilE* expressing *N. meningitidis*; however, the exact mechanisms of this regulation remains to be determined

(Kuwae *et al.*, 2011). Studies of the *N. meningitidis* proteome have implicated the RNA chaperone Hfq in pilin protein regulation. For example, deletion of *hfq* in two different class I pilin expressing meningococcal strains led to either upregulation of pilin (Fantappiè *et al.*, 2009) or no change in pilin levels (Huis in 't Veld *et al.*, 2017). In addition, RNA co-IP studies have identified Hfq-dependent sRNA which originates from 3' UTR of class I *pilE* and is co-transcribed with *pilE* in *N. meningitidis* (Heidrich *et al.*, 2017). Interestingly, class II *pilE* expressing *N. meningitidis* also contains this sRNA however it is not expressed and is present in a distinct locus, suggesting distinct roles of sRNA in class I and class II *pilE* containing isolates (Heidrich *et al.*, 2017). Although the precise function of this sRNA remains to be determined, it has been proposed to function in *trans* by integrating multiple stimuli from the environment to regulate Tfp expression and possibly pilin Av (Heidrich *et al.*, 2017).

Post-translational. The pilin protein is modified *via* glycosylation and phosphorylation and different pilin modifications can vary according to strain, pilin sequence and expression of genes responsible for post-translational modifications (PTM) (Power *et al.*, 2006). PTM are associated with particular serine residues on pilin and can impact bacterial phenotypes. For example, Ser^{63} is the target of O-linked glycosylation which is mediated by PglL. Interestingly, *N. meningitidis* Δ*pglL* mutant was not internalised by endothelial cells as efficiently as wild-type bacteria (Takahashi *et al.*, 2012). Deletion of pilin phosphotransferase B (*pptB*) that mediates the addition of phosphoglycerol to Ser^{93}, has resulted in downregulation of pilus bundling which in turn triggers bacterial detachment from microlonies and invasion of host cells (Chamot-Rooke *et al.*, 2011).

Tfp biogenesis and retraction are dependent on the regulation of different components of the Tfp machinery (Section 1.3). For example, Tfp retraction which leads to translocation of Tfp subunits into cytoplasm is linked to PilC and PilT proteins (Merz *et al.*, 2000; Morand *et al.*, 2004). In addition, the number of pili fibres on the bacterial surface correlates with the expression of *pilV* and *pilX* genes (Imhaus & Duménil, 2014). Upon interaction with host cells, Tfp have been shown to affect host cell polarity which leads to disruption of cell-cell junctions and subsequent invasion of the meninges (Coureuil *et al.*, 2009).

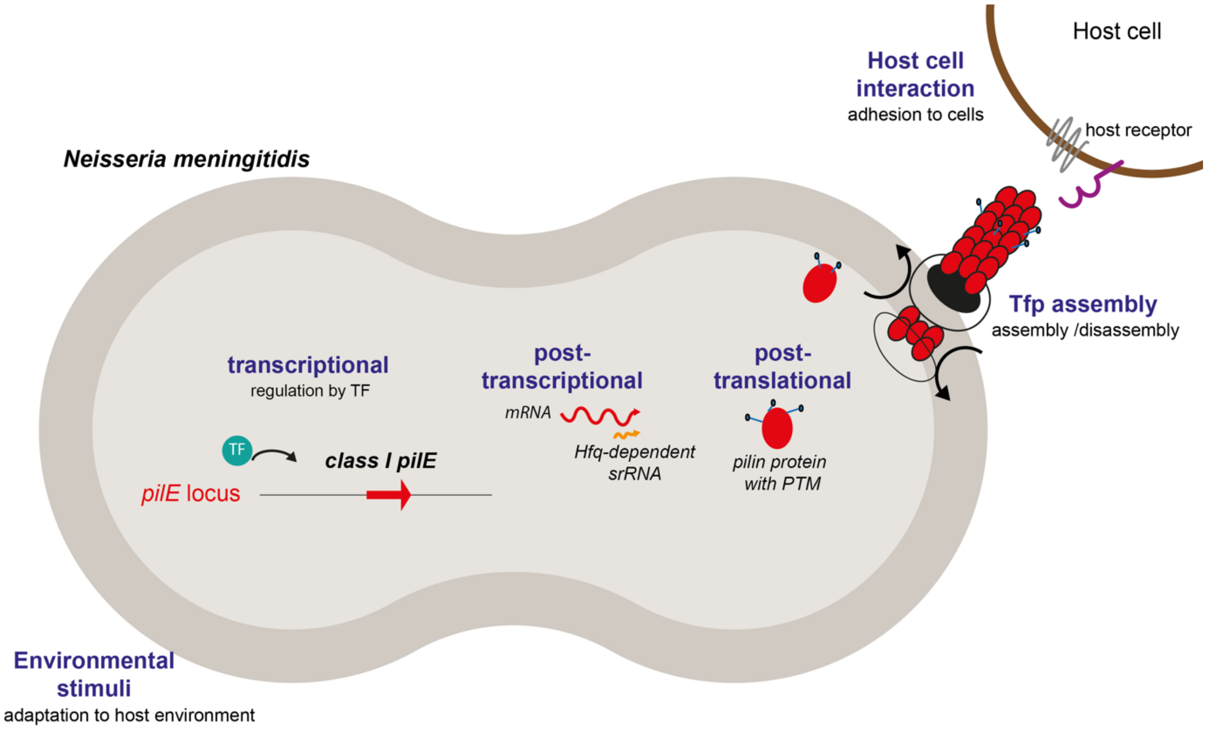


Figure 1.3 Regulation and modification of pilin in *N. meningitidis*

Mechanisms of class I *pilE* regulation in *N. meningitidis*. Different transcription factors (TF) can interact with the *pilE* upstream region regulating *pilE* at the level of transcription. Post-transcriptional regulation can take place *via* small regulatory RNA (srRNA) (Heidrich *et al.*, 2017). Pilin protein undergoes post-translational modifications (PTM) and reaches the membrane to be assembled into a fibre. Tfp fibre is regulated *via* retraction or assembly mechanisms. Tfp-mediated interaction with the host regulates bacterial adhesion. Environmental stimuli can also impact pilin expression (Larribe *et al.*, 1997).

1.5 Transcription initiation in bacteria: focus on σ factors

Since my project addresses the regulation of class II *pilE* at the level of transcription, it is important to consider how transcription is initiated in bacteria.

The initiation of bacterial transcription requires three principal components: a DNA promoter sequence, core RNA polymerase (RNAP) and sigma (σ) factors. Promoters can be composed of several different elements including upstream sequences, and either a -10/-35 or a -12/-24 sequence, which were originally defined according to their nucleotide distance from the transcription start site (Hawley *et al.*, 1983; Pribnow, 1975). RNAP is a multisubunit protein involved in promoter recognition and RNA synthesis (Hurwitz *et al.*, 1961) (Figure 1.4). It consist of α, β, β' and ω, subunits encoded by *rpoA*, *rpoB*, *rpoC* and *rpoZ* respectively (Zhang *et al.*, 1999). Each subunit mediates important functions of RNAP. The two domains of α subunit (the N- and C-terminal domains) are joined by a flexible linker region and the C-terminal domain (CTD) is engaged with the upstream promoter elements (Blatter *et al.*, 1994; Jeon *et al.*, 1995). The N-terminal domains of the α subunits interact with some transcriptional regulators, modulating the activity of the promoter. β and β' subunits constitute the catalytic core of RNAP by forming a channel through which RNA synthesis is established (Murakami *et al.*, 2002; Severinov *et al.*, 1997). β subunit engages with σ factor to initiate promoter melting and transcription initiation (Young *et al.*, 2004). The ω subunit is the smallest subunit of RNAP and has been implicated in the assembly of the β' subunit (Ghosh *et al.*, 2001), and there is evidence that it can modulate RNAP activity in response to external stimuli (Periago *et al.*, 2002). Furthermore, the ω subunit has also been shown to play a role in the stringent response: crosslinking experiments as well as RNAP mutant analysis revealed that ppGpp, which is one of the key signalling molecules during stringent response, binds to ω and β' subunits thereby affecting gene expression (Ross *et al.*, 2013).

The first crystal structure of core RNAP was obtained from *Thermus aquaticus* which demonstrated that RNAP contains "crab-claw" structure (Zhang *et al.*, 1999). The studies revealed that β and β' subunits are connected by the αNTDs and form two "jaws" or clamps with the cleft in between. This multisubunit arrangement is dynamic and can adopt different conformational states during transcription initiation, elongation and transcription termination (Darst *et al.*, 2002). The cleft can be separated into several channels including a) primary channel (involved in dsDNA and RNA/DNA hybrid interaction), b) secondary channel (NTP entry for RNA synthesis) and c) RNA exit channel (associated with RNA/DNA hybrid strand separation) (Lee *et al.*, 2016; Vassylyev *et al.*, 2007). RNAP engages with σ factor which facilitates the efficient interaction between σ domains and the corresponding promoter sequences (Mekler *et al.*, 2002). σ^{70} contains four conserved domains ($\sigma_{1\text{-}4}$) and the crystal structure of RNAP and σ holoenzyme revealed that the core surface of the RNAP is bound by the domains $\sigma_{1\text{-}3}$ (Murakami, 2013). The linker between domains 3 and 4 is positioned within primary channel and blocks the RNA exit channel. Domain 4 in turn occupies the cleft between β and β' subunits and obstructs the primary channel. Upon transition from transcription initiation to elongation, the interactions between σ factor and RNAP weaken as nascent RNA displaces the linker and domain 4 (Nickels *et al.*, 2005).

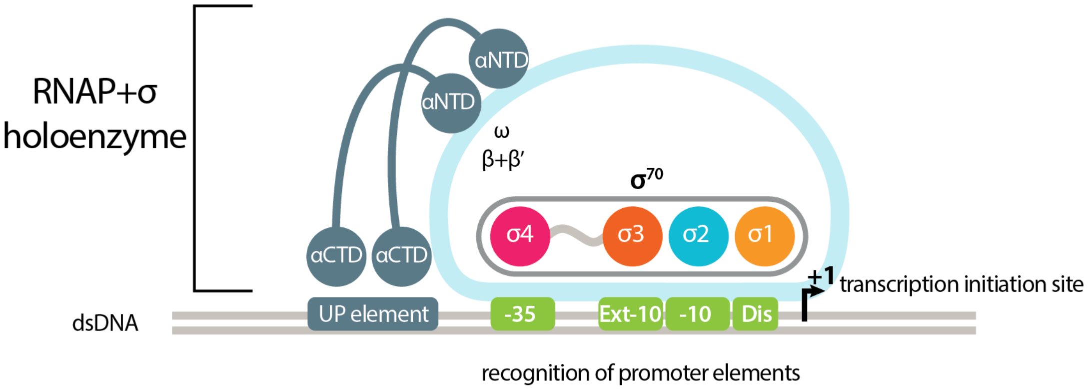


Figure 1.4 RNA polymerase-σ promoter interaction

Schematic diagram showing the subunit structure of RNA polymerase-σ factor holoenzyme and its interaction sites with a promoter region. σ^{70} factor contains four domains which recognise distinct promoter elements: -35, extended -10, -10 and discriminator region (Dis). RNA polymerase is comprised of α, β, β' and ω subunits. αCTDs recognise the upstream (UP) element present at some promoters and β and β' form the catalytic core of the enzyme whereas the ω subunit assists the folding of the ββ' subunits. The transcription initiation site (+1) is indicated with an arrow.

1.5.1 Bacterial σ factors

Core RNAP must associate with a σ factor to form the RNAP holoenzyme that recognises specific promoter sequences and initiates transcription. Transcription is initiated by σ-dependent strand separation of dsDNA and formation of transcription bubble (Feklístov *et al.*, 2014). Bacteria express different σ factors some of which are responsible for activating a subset of genes in response to stimuli, allowing bacteria to adapt to changing environments. Based on studies in *E. coli*, σ factors can be divided into two structurally different families: the σ^{70} and σ^{N} family.

1.5.1.1 σ^{70} family

σ factors belonging to σ^{70} family recognise consensus promoter elements known as -10 and -35 sequences. The σ^{70} family can be divided into four groups according to their modular structure (Figure 1.5A) (Helmann *et al.*, 1988). All four groups share some similarities in the structure of the promoter-binding domains (Gruber *et al.*, 2003). Domains 2 and 4 are present in all four groups of σ factors. Domain 2 is responsible for binding -10 promoter sequence and discriminator motif. Domain 4 in turn is involved in recognition of -35 promoter sequence *via* helix-turn-helix (HTH) motif. In addition to the two conserved DNA binding domains, Groups 1, 2 and 3 of σ^{70} contain domain 3, which is an α helix that interacts with extended -10 sequence of the promoter (Feklistov *et al.*, 2011). Strand separation occurs when domain 2 of a σ factor recognises -10 promoter sequence and captures nucleotides within the -10 motif. The specific bases are flipped from -10 region and are inserted into defined pockets within σ and transcription is initiated (Zhang *et al.*, 2012). Base specificity as well as the number of bases that are captured from the nontemplate strand by domain 2 differs among σ^{70} family Groups.

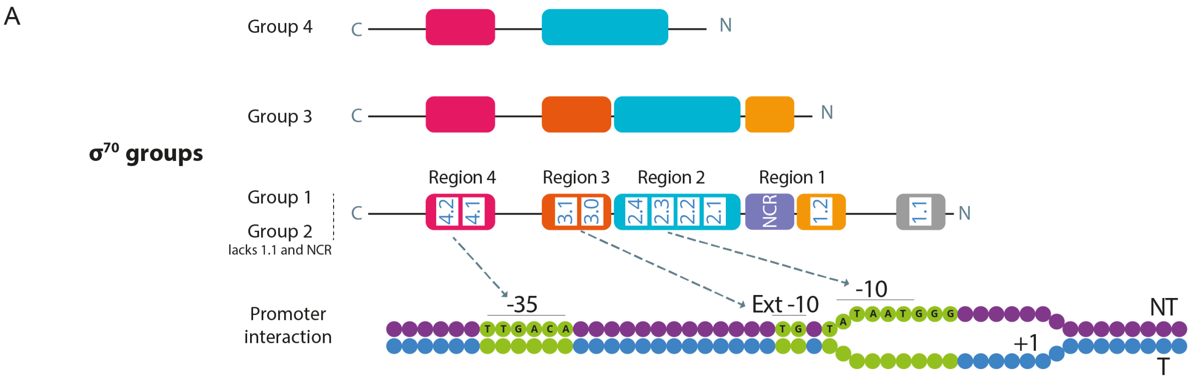
Group 1 contains the major essential *E. coli* σ^D factor encoded by *rpoD* gene, which is known as the 'housekeeping σ factor' and is referred to as σ^A in other bacterial species. σ^D is responsible for basal gene transcription in the majority of species (Feklístov *et al.*, 2014). It consists of four domains, each with a distinct function. Domain 1.1 ($\sigma_{1.1}$), which is present only in Group 1 σ factors, acts as the control mechanism for unwanted transcription by inhibiting the entry of non-specific DNA elements into the active site of the polymerase. Unlike other σ factors, σ^D can recognise a diverse range of -10/-35 promoters and has inherently low specificity. Domains 2 and 4 are the two largest RNAP interacting domains that make direct contact with the -10/-35 promoter. Domain 4 establishes the first contact between the σ-RNAP complex *via* HTH motif and the double stranded -35 sequence (deHaseth *et al.*, 1998). Domain 2 of σ^{70} is involved in recognition of -10 element ($T_{12}A_{11}T_{10}A_{9}A_{8}T_{7}$) upon strand separation (Feklistov *et al.*, 2011). Domain 2 captures two bases within -10 element: A_{11} and T_{7} in the nontemplate strand (Feklistov & Darst, 2011). A_{11} and T_{7} nucleotides are extruded from dsDNA and are buried in the pockets within domain 2, which leads to transcription bubble formation. The presence of two protein pockets increases the DNA melting capacity of Group 1 σ factors allowing σ^{70} factors to recognise a wide range of -10/-35 promoter sequences (Feklístov *et al.*, 2014). Domain 3 located between domains 2 and 4 is responsible for interactions with the extended -10 promoter sequence (5'-TG-N-TATAAT-3') (Barne *et al.*, 1997). Domain 3 recognition of the extended -10 region contributes to the stability of the holoenzyme, which is crucial as the extended -10 is thought to enhance RNAP-promoter specificity in the absence of a consensus -35 sequence (Tang *et al.*, 2005).

Group 2 in *E. coli* comprises the σ^{S} factor encoded by *rpoS* gene, which is a stress response regulator originally identified as the σ factor involved in maintaining stationary phase gene expression (Lange *et al.*, 1991). Group 2 σ factors are highly homologous in structure to the Group 1 σ^{70} except for Region 1.1. The lack of Region 1.1 decreases the specificity of σ^{S} for the promoter, resulting in σ^{S} being able to initiate transcription from non-consensus -10/-35 promoters (Typas *et al.*, 2007).

Group 3 σ factors can be subdivided into four phylogenetically divergent subfamilies: 1) σ^F encoded by *rpoF*, which is involved in flagellar biosynthesis, 2) σ^H encoded by *rpoH* which is the heat shock response regulator, 3) σ^B present in *B. subtilis* and involved in general stress response and 4) σ^F, σ^E, σ^G and σ^K which orchestrate sporulation in *B. subtilis*. Unlike other groups, each Group 3 σ factor relies on a defined promoter sequence that is associated with a specific σ factor (Figure 1.5B). The sequence of each promoter element as well as the spacing between promoter regions is crucial for promoter recognition. For example, promoters that lack a defined σ^H specific -10/-35 sequence (CCCCATNT-N_{15}-TTGAAA) are either not recognised by σ^H or recognition leads to only partial activation (Nonaka *et al.*, 2006). Group 3 σ factors are upregulated in response to a specific stress and therefore control a defined sub-set of genes that are necessary to be activated only under certain conditions. A requirement for a unique promoter makes these σ factors more specific in orchestrating the appropriate response.

Group 4 includes more than 40 distinct sub-clades of σ factors which are collectively known as extracytoplasmic function factors (ECF) (Anna *et al.*, 2009). *E. coli* ECF σ factors include the envelope stress regulator σ^E encoded by *rpoE* and ferric citrate uptake σ^{FecI} encoded by *fecI*. ECFs share little sequence identity with other σ factors as they only contain two domains: σ_2 and σ_4, restricting the number of promoters they recognise.

Unlike domain 2 of Group 1 σ factors, domain 2 of Groups 3 and 4 contains single pocket and captures only one base within the nontemplate strand of the -10 element (Campagne *et al.*, 2014). This decreases the melting capacity and limits the number of promoters that can be recognised by these σ factors (Koo *et al.*, 2009).



B

σ factor	Consensus promoter sequence			Function	Group
E. coli					
σ^D/σ^{70}	TTGACA	- 17nt -	TATAAT	Housekeeping genes	1
σ^E/σ^{24}	GAACT	- 17nt -	TCTGAt	Extracytoplasmic stress response	4
σ^H/σ^{32}	TTGAAA	- 15nt -	CCCCATNT	Heat shock response	3
σ^F/σ^{28}	TAAAgt	- 15nt-	GCCGATAA	Regulation of motility genes	3
σ^S/σ^{38}	TTGACA	- 17nt -	TATACT	Stationary phase response	2
$\sigma^{FecI}/\sigma^{19}$	aaAAT	- 17nt -	TATAAT	Extracytoplasmic stress response	4
N. meningitidis					
σ^{70}	consensus absent consensus absent	- not defined -	TGNTATAAT TATAAT	Housekeeping genes	unknown
σ^E/σ^{24}	TtGaACTT	- 15nt -	TATAAT	Extracytoplasmic stress response	unknown
σ^H/σ^{32}	GTTGAA	- 20nt -	TAtAAT	Heat shock response (defined in *N. gonorrhoeae*)	unknown

Figure 1.5 The σ^{70} family

A. Four groups of σ^{70} family sigma factors and their respective domain organisation: Region 4 (pink), Region 3 (orange), Region 2 (blue) and Region 1 (pale orange and grey). Region 4, 3 and 2 mediate interaction with -10/-35 and extended -10 DNA sequences (shown in green). B**.** σ factors present in *E. coli* and *N. meningitidis* with the corresponding promoter consensus sequences and proposed function (adapted from (Bervoets *et al.*, 2018; Paget, 2015)

1.5.1.2 σ^{70} family sigma factors in *N. meningitidis*

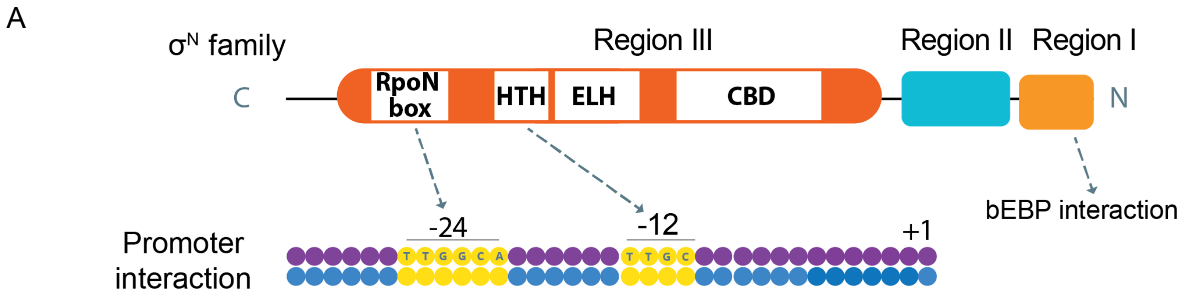
Within the host, *N. meningitidis* encounters a number of different environments to which it needs to adapt and trigger appropriate gene expression necessary for survival (Laver *et al.*, 2015). *N. meningitidis* contains three genes encoding σ^{70} family proteins: *rpoD*, *rpoE* and *rpoH* (Figure 1.5B). σ^{H} and σ^{E} have been shown to be functional (Du *et al.*, 2005) and based on the published work only σ^{E} regulon has been defined in *N. meningitidis* (Huis in 't Veld *et al.*, 2011). The σ^{H} regulon has been studied in the related pathogen *N. gonorrhoeae*. Nearly all the genes in the gonococcal σ^{H} regulon are classified as heat shock response genes, suggesting that in *N. gonorrhoeae*, σ^{H} may contribute to adaptation to increased temperature (Gunesekere *et al.*, 2006). σ^{H} has also been shown to be essential in *N. meningitidis* at 37°C (Capel *et al.*, 2016), unlike in *E. coli*, where σ^{H} is only essential at elevated temperatures (>42°C) (Yura *et al.*, 1993). This indicates different contributions of the σ factors in the two organisms. Genome wide analysis of transcriptional start sites identified three different types of σ^{70} family dependent-promoters in *N. meningitidis*: two types of σ^{70} factor-dependent promoters upstream of 458 genes which lack the consensus -35 element, and putative σ^{E}-dependent promoters upstream of 38 genes (Figure 1.5B) (Heidrich *et al.*, 2017). However, experimental validation of the proposed promoter types was not performed. No σ^{H}-dependent promoters were identified in *N. meningitidis*, suggesting that either σ^{H} also recognises σ^{70} and σ^{E}-dependent consensus sequences or that *N. meningitidis* possesses novel σ^{H}-dependent promoter sequences.

1.5.1.3 σ^{70} family-dependent transcription initiation

There are multiple steps in transcription initiation by σ^{70} family-RNAP holoenzymes. σ factors on their own are incapable of interacting with promoter elements due to their conformational fold. Therefore σ^{70} family factors must interact with RNAP in order to bind to their -10/-35 promoter sequence. First, the σ^{70}-RNAP holoenzyme forms the closed complex (RPc) (Saecker *et al.*, 2011). Interaction between σ factor and RNAP leads to conformational change within the complex, which can recognise and bind the DNA promoter. The recognition of the dsDNA promoter elements by domain 2 of σ factor is coupled to the opening of the DNA helix and transcription bubble formation (Feklistov & Darst, 2011). Subsequently the stable open complex is formed (RPo) and the template strand is loaded into the active site cleft of RNAP for elongation to occur (Gries *et al.*, 2010). RNAP holoenzyme has recently been shown to undergo a series of motions during transcription initiation. Upon promoter recognition, the RNAP clamp temporarily closes which leads to RNAP docking to the promoter DNA and dsDNA opening. The clamp opens and ssDNA loading occurs which is followed by clamp closure to ensure that DNA melting is completed and transcription can proceed (Feklistov *et al.*, 2017). During elongation, RNAP catalyses the synthesis of nascent RNA which competes with domains 3 and 4 of σ factors. As RNA reaches ~16 nt and extends through the RNA exit channel, σ^{70} interactions with DNA and RNAP are broken and σ^{70} undergoes stochastic dissociation from RNAP leading to promoter clearance (Murakami *et al.*, 2002; Raffaelle *et al.*, 2005). Once dissociated, σ factor becomes free to engage with another RNAP for subsequent transcription initiation.

1.5.1.4 σ^N family sigma factors

A distinct class of σ factors known as σ^N (or σ^{54}) factors are encoded by *rpoN*. σ^N recognises a specific promoter known as the -12/-24 promoter. Originally -12/-24 consensus sequence was defined as -12 (GC)/ -24 (GG) based on the DNA footprinting assays that showed σ^N-dependent protection of these residues (Morett *et al.*, 1989). Subsequently the alignment of 186 known -12/-24 promoters with the defined transcriptional start site from a number of different bacterial species revealed additional highly conserved nucleotides adjacent to conserved -12/-24 (Barrios *et al.*, 1999). Together with mutagenesis analysis of different -12/-24 promoters, an updated -12/-24 consensus sequence was established: TGGCW (-24)-N4-YTGGCACG (-12) where N is any nucleotide, Y is a pyrimidine and W is A or T (Figure 1.6A) (Barrios *et al.*, 1999). σ^N is comprised of domains which are structurally distinct to those found in σ^{70} family factors (Figure 1.6A). Region I located at the N-terminus mediates interactions with the activator proteins known as bacterial enhancer binding proteins (bEBP), which are essential for σ^N-dependent transcription (Popham *et al.*, 1989). In the absence of the activator, Region I interacts with Region III to occupy the active site between β and β' subunits. This prevents the entry of the DNA into the RNAP holoenzyme and blocks σ transcription initiation. Region II is positioned within the DNA binding channel of the RNAP holoenzyme and can be further divided into three subregions: 1) Region II.1 plays an inhibitory role by occupying the DNA binding site of the cleft, 2) Region II.2 is located within the active cleft where DNA template strand is loaded thereby preventing the entry of the template strand into RNAP active cleft, and 3) Region II.3 is present within the region of RNAP that would otherwise be occupied by nascent RNA during transcription (Yang *et al.*, 2015; Glyde *et al.* 2017). Both Regions II.2 and II.3 have to be displaced to allow transcription initiation and elongation to take place. Region III is conserved and contains both RNAP interacting core binding domain (CBD), extra long α-helix (ELH) domain and two key DNA-binding motifs: a Helix Turn Helix (HTH) motif and a characteristic RpoN box (Cannon *et al.*, 1995; Wong *et al.*, 1994) (Figure 1.6A). Recent crystal structures of *E. coli* σ^N-RNAP holoenzyme revealed that the RpoN box is a flexible domain involved in interaction with the -24 sequence (Yang *et al.*, 2015). The HTH motif in turn forms stable interaction with RNAP core and mediates the binding and recognition of -12 promoter element (Merrick *et al.*, 1992; Taylor *et al.*, 1996; Yang *et al.*, 2015). σ^N mutants harbouring mutations within the HTH region maintain no -12 promoter contacts while still retaining -24 interaction (Wang *et al.*, 1999; Wong *et al.*, 1994). The opposite is observed when the HTH motif is left intact but the RpoN box is mutated: in this case the binding of σ^N is below 20% of the wild-type, suggesting that for σ^N promoter binding, the interaction between σ^N and the -24 element is more important than the σ^N interaction with the -12 element (Hsieh *et al.*, 1994; Wang *et al.*, 1999).


A
σ^N family
Region III
Region II
Region I
C
N
RpoN box
HTH
ELH
CBD
bEBP interaction
Promoter interaction
-24
-12
+1
T T G G C A
T T G C

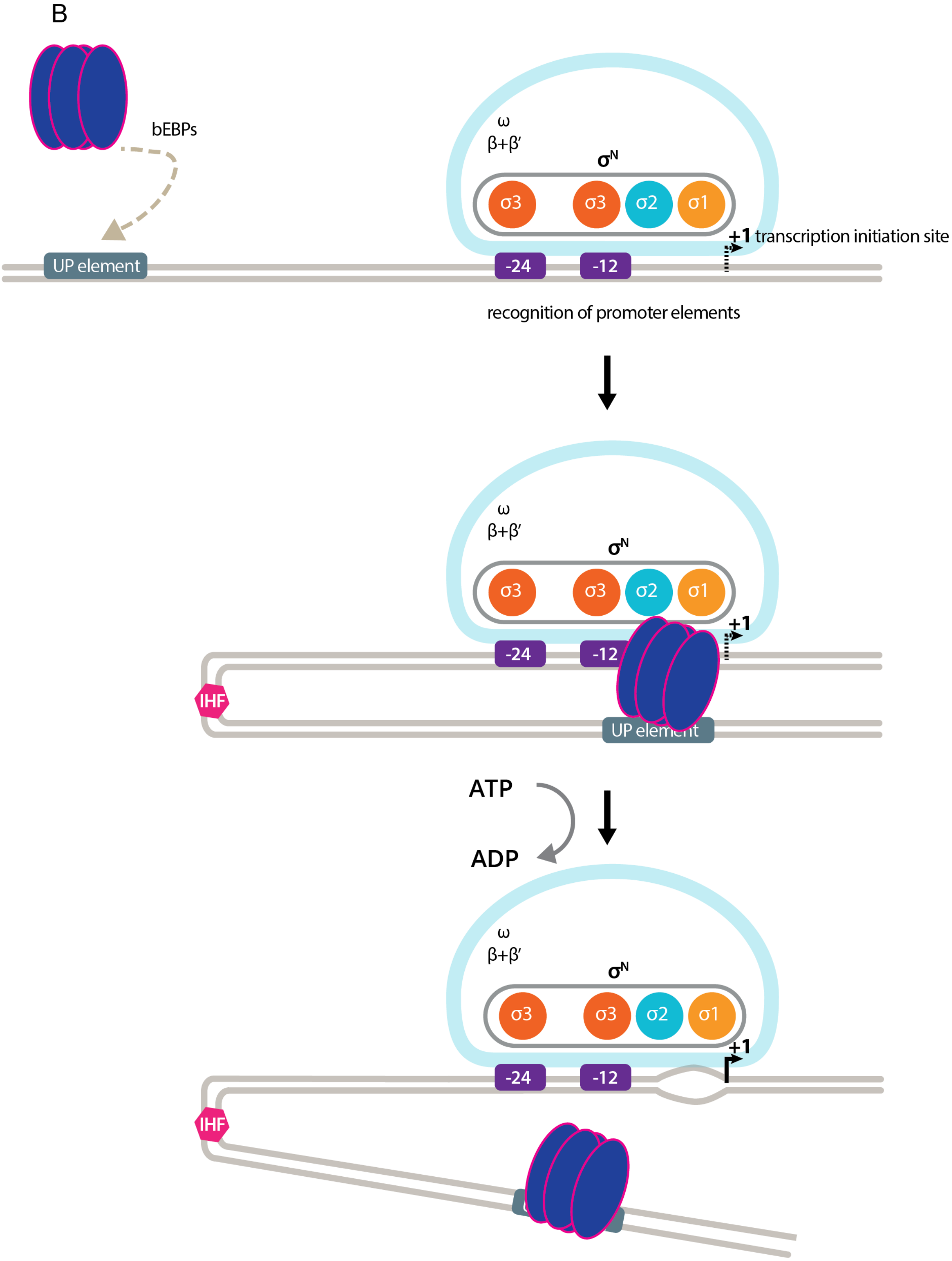

B
bEBPs
UP element
ω
β+β′
σN
σ3
σ3
σ2
σ1
+1 transcription initiation site
-24
-12
recognition of promoter elements
ω
β+β′
σN
σ3
σ3
σ2
σ1
+1
-24
-12
IHF
UP element
ATP
ADP
ω
β+β′
σN
σ3
σ3
σ2
σ1
+1
-24
-12
IHF

Figure 1.6 σ^N-dependent transcription

A. Diagram representing σ^N domains: Region III (red), Region II (blue) and Region I (orange). DNA binding regions within Region III that are responsible for -12/-24 promoter recognition include the RpoN box and HTH. Additional domains within Region III are extra long helix (ELH) and core binding domain (CBD). B. σ^N recognises -12/-24 promoters. RNAP and σ^N form a closed complex at the promoter site. DNA binding proteins, for example integration host factor (IHF), bind the upstream region of the promoter and initiate DNA bending bringing bEBP oligomers in close proximity to the holoenzyme. The bEBP hexamer triggers ATP-dependent conformational change within the holoenzyme that leads to formation of the open complex, DNA melting and transcription.

The mechanism of promoter recognition and transcription initiation involves transition between the closed complex (RPc) and open complex (RPo). Recent work by Zhang and colleagues revealed the “coupled DNA loading and promoter melting” model of σ^N-dependent transcription initiation, which is similar to transcription initiation triggered by σ^{70}-RNAP holoenzyme (Glyde *et al.*, 2018). σ^N can recognise some -12/-24 elements in the absence of RNAP (Buck *et al.*, 1992). When RNAP engages with σ^N, the holoenzyme resides at the double stranded -12/-24 sequence in a closed complex state unable to initiate transcription. Within the RPc DNA is positioned between β and β' subunits and the clamp remains closed. The transient opening of the clamp occurs during subsequent formation of two intermediate complexes (RPi) (Glyde *et al.*, 2018). RPi complexes are characterised by the opening of the clamp, which allows DNA to be loaded in the holoenzyme. During the transition between RPi and RPo the clamp closes again which ensures that DNA is positioned within the active site cleft and strand separation occurs. RPo is stabilised by extra long helix motif within Region III of σ^N, which physically separates the DNA strands and does not involve any base capturing from promoter element seen in σ^{70}-dependent transcription. The conformational changes within σ^N-RNAP holoenzyme during transcription initiation are facilitated by bacterial enhancer binding proteins (bEBP). bEBPs engage with Region I of σ^N removing the inhibition of transcription. bEBPs are ATPase containing AAA+ family proteins which are activated in response to a given stimulus. They bind to upstream activator sequences (UASs) situated 80-150 bp upstream of the -12/-24 sequence (Bush *et al.*, 2012), where they oligomerise. Oligomerised bEBPs are brought into close proximity to the holoenzyme *via* a DNA looping mechanism, which is frequently facilitated by proteins such as integration host factor (IHF) (Hoover *et al.*, 1990). Interaction between bEBP and the holoenzyme promotes stand separation and subsequent transcription bubble formation by weakening the dsDNA downstream of -12 promoter element (Glyde *et al.*, 2017).

Previous studies of σ^N in *Neisseria* spp. have shown that although *rpoN* is present, σ^N is non-functional in *N. gonorrhoeae* due to a frameshift mutation that results in truncation and loss of the HTH motif and RpoN box (Laskos *et al.*, 1998; Rendon *et al.*, 2013). In addition, the failure to detect σ^N in *N. meningitidis* and *N. gonorrhoeae* using a mAb raised against *S. typhimurium* RpoN (Laskos *et al.*, 1998) as well as the lack of transcriptional activity from the σ^N-dependent pilin promoter strongly suggests that this σ factor is non-functional in *N. meningitidis* and *N. gonorrhoeae*. In the non-pathogenic *N. elongata*, the *rpoN* gene encodes a protein containing both DNA binding motifs which can bind the *pilE* promoter and activate *pilE* transcription (Rendon *et al.*, 2013). In *N. meningitidis*, *rpoN* encodes a truncated σ^N, which lacks an obvious HTH motif, but retains the RpoN box (Rendon *et al.*, 2013). This will be discussed further in Chapter 4.

1.6 Regulation of transcription initiation by different σ factors

Under steady state the number of RNAP molecules present is relatively constant, leaving the σ factors to compete for the limited RNAP pool (Jishage *et al.*, 1995). σ^D has the highest affinity for RNAP and alternative σ factors must therefore displace σ^D from the RNAP core to form their own σ-RNAP complexes to activate transcription. Several mechanisms exist to overcome σ^D-dependent RNAP interactions, such as increasing in the number of alternative σ factor molecules in the cell (Straus *et al.*, 1987). For example, in *E. coli* the heat shock response regulator σ^H is complexed with the anti-σ^H which keeps it in the inactive state under growth at 'normal' temperature (Lim *et al.*, 2013; Miyazaki *et al.*, 2016). Following exposure to elevated temperature, anti-σ^H factor releases σ^H therefore transiently increasing the concentration of σ^H molecules in the cell, which enables the σ^H to sequester more RNAP and activate transcription of the genes required to adapt to the environmental conditions (Guisbert *et al.*, 2004; Mauri *et al.*, 2014). Alternatively, the direct interaction of proteins with σ^D can obstruct σ^D interactions with RNAP. This in turn results in increase in the unbound RNAP molecules that can be associated with alternative σ factors, thereby increasing transcription of alternative σ factor regulons (Sharma *et al.*, 2010). An example of this is Rsd which in *E. coli* is able to sequester σ^D when bacteria enter stationary phase (Jishage *et al.*, 1999; Piper *et al.*, 2009). Rsd binding to σ^D reduces the competition between σ^D and σ^S, which is the key regulator of the stationary phase response, thereby allowing σ^S to interact with RNAP and initiate transcription from σ^S-dependent promoters.

In many organisms a number of genes have more than one promoter, leading to complex patterns of regulation (Ishihama, 2010). Multiple promoters can act synergistically thereby enhancing transcription, and/or can be activated in response to different environmental stimuli. On the other hand, multiple promoters may have antagonistic effects, whereby the binding of RNAP to one promoter may obstruct transcriptional activation from the others (Zafar *et al.*, 2014). Furthermore, evidence suggests that under different conditions σ cross talk may occur and multiple σ factors can recognise the same promoter sequences (Cho *et al.*, 2014; Wade *et al.*, 2006). A ChIP-seq analysis of all six σ factors in *E. coli* (σ^{70} family members and σ^{N}) demonstrated that many promoter regions can be bound by several different σ factors (Cho *et al.*, 2014). For instance, more than 800 binding sites were shown to interact with both σ^{D} and σ^{S} in *E. coli*. However, the difference in the promoter activity in the presence of each σ factor has not been studied (Cho *et al.*, 2014). Thus, transcriptional activation is a complex and multifactorial process, which can be regulated at multiple levels, and which is fundamental to modulation of gene expression.

1.7 Project aims

The aim of my thesis work was to characterise the promoter of the class II *pilE* gene of *N. meningitidis*. The *pilE* promoter in meningococci that express variable class I pilin has been studied previously, and class I *pilE* transcription has been shown to initiate from a σ^{70}-dependent promoter, despite the presence of multiple putative promoters including a σ^{N} binding site. The region upstream of the class II *pilE* gene in *N. meningitidis* also contains multiple putative promoters: extended -10 sequence and -10/-35 (both σ^{70} family dependent), and -12/-24 (σ^N-dependent) promoters (Figure 1.7). The sequence and relative position of these promoters differs compared to the promoter of the class I *pilE* gene suggesting that invariant class II pilins may be subject to different transcriptional regulation.

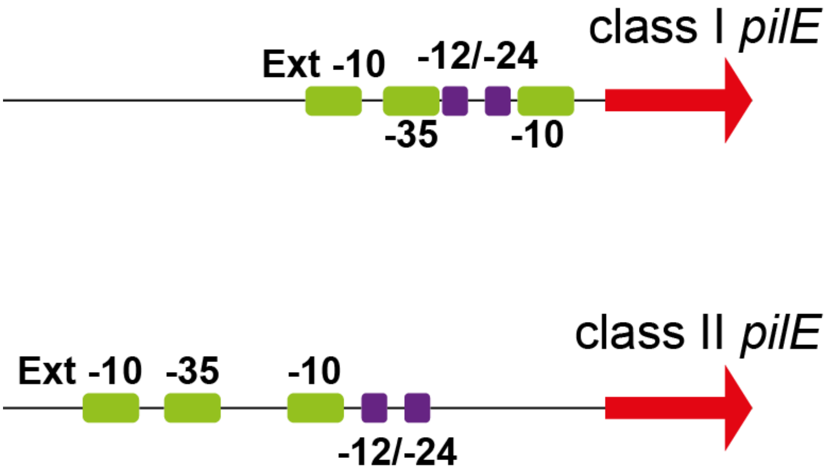


Figure 1.7 Schematic diagram of class I and class II *pilE* promoters

Three putative promoter sequences were identified upstream of *pilE*: an extended -10 and -10/-35 (green) and -12/-24 (purple). The latter has different position in class II compared to class I pilin strains.

My project aimed to identify the promoter(s) of the class II *pilE* gene and to determine the contribution of transcription factors and σ factors to class II pilin expression. Specific aims were as follows:

i) Analysis of the conservation of *pilE* promoter sequences in meningococcal isolates

ii) Experimentally define the contribution of the different promoter elements to class II *pilE* transcription

iii) Investigate the conservation and roles of σ factors in *N. meningitidis*.

2 Materials and Methods

2.1 Antibiotics

Table 2.1 Antibiotics used in this work

Antibiotics were used at the final concentration:

Antibiotics	Concentration for *E. coli*	Concentration for *N. meningitidis*
kanamycin	50 µg/ml	75 µg/ml
carbenicillin	100 µg/ml	n/a
chloramphenicol	30 µg/ml	n/a
erythromycin	n/a	2 µg/ml

2.2 Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 2.3. *E. coli* was grown at 37^{o}C, supplemented with antibiotics as described in Table 2.1 where appropriate, on either Luria-Bertani (LB) agar, or in 5 ml LB liquid with shaking at 180 rpm. *N. meningitidis* was grown at the desired temperature, 5% CO_2 on brain heart infusion (BHI, Oxoid) agar supplemented with 0.1% (w/v) starch, 5% (w/v) heat denatured horse blood and antibiotics (Table 2.1) as appropriate, or in BHI liquid. To inoculate liquid cultures, *N. meningitidis* was grown overnight on BHI agar. A loop of bacteria was harvested from the plate and resuspended in 600 µl of PBS, and DNA was quantified in the lysis buffer (P2 buffer, Qiagen) using A_{260nm}. A total of 10^9 cfu were used to inoculate 25 ml of BHI in 125 ml conical flask (Corning), and then were incubated at appropriate temperatures with shaking at 180 rpm. The OD_{600} of cultures was used to monitor growth.

2.2.1 Plasmids used in this study

Table 2.2 Plasmids used in the study

Plasmid	**Description**	**Source**
pGEM-TEasy	Cloning and expression vector, carbenicillin resistance	Promega
pGEMT-Easy-Δ*rpoN*	pGEM-TEasy harbouring construct for deletion of *rpoN* in *N. meningitidis* strain S4, kanamycin resistance	This study
pGEM-Easy-*rpoN*-His	Vector harbouring construct for C-terminal His addition to *rpoN* in N. *meningitidis* S4	This study
pCONJ4S	Conjugation vector, for construction of markerless *E. coli* mutants, contains GFP, carbenicillin resistance	Tang lab, unpublished
pKO3	Vector for construction of markerless *E. coli* mutants, temperature sensitive, chloramphenicol resistance	Link *et al*., 1997
pCR2.1-TOPO	Cloning and expression vector, ampicillin resistance	Invitrogen
pCR2.1-TOPO-Δ*rpoN*	Vector harbouring construct for *rpoN* deletion in *N. meningitidis* strain S4, erythromycin resistance	This study
pCR2.1-TOPO-Δ*pilE*P*pilE*-*lacZ*	Vector harbouring construct for *rpoN* deletion and *pilE* ORF replacement with *lacZ* in *N. meningitidis* strain S4	This study
pUC19	Cloning vector, carbenicillin resistance	Yanisch-Perron et *al*., 1985
pUC19-Δ*rpoE*	Vector harbouring construct for *rpoE* deletion in *N. meningitidis* strain S4	This study
pUC19-*rpoH*$^{\mathrm{FLAG}}$	pUC19 harbouring *rpoH* from *N. meningitidis* with C terminal FLAG tag	This study
pRS415	Vector harbouring promoterless *lacZYA* operon for promoter- *lacZ* fusions, carbenicillin resistance	Simons *et al*., 1987
pRS415-P1*pilE*	Vector harbouring 153 bp of the upstream region of *pilE* from *N. meningitidis* FAM18	This study

pRS415-P2*pilE*	Vector harbouring 199 bp of the upstream region of *pilE* from *N. meningitidis* FAM18	This study
pRS415-P3*pilE*	Vector harbouring 322 bp of the upstream region of *pilE* from *N. meningitidis* FAM18	This study
pRS415-P*pspA*	Vector harbouring 170 bp of the upstream region of *pspA* from *E. coli* MG1655	This study
pBAD33	Expression vector, kanamycin resistance	Guzman *et al.*, 1995
pBAD33-*rpoN*$^{E.coli}$	pBAD33 harbouring codon-optimised *rpoN* from *E. coli* MG1655	This study
pBAD33-*rpoN*^{Nm}	pBAD33 harbouring codon-optimised *rpoN* from *N. meningitidis* S4	This study
pBAD33-*rpoN*$^{E.coli}$-HA	pBAD33 harbouring codon-optimised *rpoN* from *E. coli* MG1655 with a C terminal HA tag	This study
pBAD33-*rpoN*^{Nm}-HA	pBAD33 harbouring codon-optimised *rpoN* from *N. meningitidis* S4 with a C terminal HA tag	This study
pNMC2	*N. meningitidis* S4 complementation vector for integration at *iga-trpB* locus, erythromycin and kanamycin resistance	This study
pNMC2-*rpoN*^{Nm}-FLAG	Vector harbouring *rpoN* from *N. meningitidis* with a C terminal FLAG tag	This study
pNMC2-*rpoE*^{Nm}-FLAG	Vector harbouring *rpoE* from *N. meningitidis* with a C terminal FLAG tag	This study
pNMC2-*rpoH*^{Nm}-FLAG	Vector harbouring *rpoH* from *N. meningitidis* with a C terminal FLAG tag	This study
pNMC2-*rpoN*^{Nel}	Vector harbouring wild type *rpoN* from *N. elongata* 29315	This study
pNMC2-*rpoN*^{Nm}	Vector harbouring wild type *rpoN* from *N. meningitidis* S4	This study
pNMC2-*rpoE*^{Nm}	Vector harbouring wild type *rpoE* from *N. meningitidis* S4	This study
pNMC2-*rpoH*^{Nm}	Vector harbouring wild type *rpoH* from *N. meningitidis* S4	This study

Table 2.3 Bacterial strains used in this study

Strain	**Genotype/Description**	**Source**
E. coli		
MG1655	F- λ-ilvG-rfb-50 rph-1	Lab collection
BL21	B F$^-$ *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λ(DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*]) [*malB*$^+$]$_{\mathrm{K\text{-}12}}$(λ^S)	Lab collection
Dh5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^-m_K^+$), λ$^-$	Lab collection
SM10 λ*pir*	(F$^-$) RP4-2-Tc::Mu *recA* λ*pir* lysogen Km^r	Simon *et al.*, 1983
MG1655Δ*rpoN*	MG1655 with a markerless *rpoN* deletion, glutamine auxotroph (due to *rpoN* deletion)	This study
MG1655Δ*lacZ*Δ*rpoN*	MG1655 with markerless *rpoN* and *lacZ* deletion, glutamine auxotroph (due to *rpoN* deletion)	This study

N. meningitidis		
FAM18	Serogroup C, ST-11 complex/ET-37, class II *pilE*	Lab collection
8013	Serogroup C, ST-18, class I *pilE*	Lab collection
S4	Serogroup C, ST-11 complex/ET-37, class II *pilE*	Uria *et al.*, 2008
S4Δ*rpoN*	Kanamycin resistance cassette replacing *rpoN* gene	This study
S4Δ*rpoN::Ery*	Erythromycin cassette replacing *rpoN* gene	
S4Δ*rpoN*Δ*pilE*::P*pilE-lacZ*	Erythromycin cassette replacing *rpoN; lacZ* replacing *pilE* gene	This study
S4Δ*pilE*::P*pilE-lacZ*	*lacZ* replacing *pilE* gene	This study
S4Δ*rpoE*	Erythromycin cassette replacing *rpoE gene*	This study
S4Δ*pilE*	Kanamycin cassette replacing *pilE* gene	This study

S4P$_{\text{lac}}$*Nel* $\sigma^{\text{N-FLAG}}$	*rpoN* from *N. elongata* strain 29315 fused to a 3' FLAG (DYKDHDGDYKDHDIDYKDDDDK) tag at *iga-trpB* intergenic locus in *N. meningitidis* S4. Erythromycin resistant	This study
S4P$_{\text{lac}}$*Nel* σ^{N}	*rpoN* from *N. elongata* strain 29315 at *iga-trpB* intergenic locus in *N. meningitidis* S4. Erythromycin resistant	This study
S4P$_{\text{lac}}$*Nm* $\sigma^{\text{N-FLAG}}$	*rpoN* from *N. meningitidis* S4 fused to a 3' FLAG at *iga-trpB* intergenic locus in *N. meningitidis* S4. Erythromycin resistant	This study
S4P$_{\text{lac}}$*Nm* σ^{N}	*rpoN* from *N. meningitidis* S4 at *iga-trpB* intergenic locus in *N. meningitidis* S4. Erythromycin resistant	This study
S4P$_{\text{lac}}\sigma^{\text{E-FLAG}}$	*rpoE* from *N. meningitidis* S4 fused to a 3' FLAG at *iga-trpB* intergenic locus. Erythromycin resistant	This study
S4P$_{\text{lac}}\sigma^{\text{E}}$	Wild type *rpoE* from *N. meningitidis* S4 at *iga-trpB* intergenic locus. Erythromycin resistant	This study
S4P$_{\text{lac}}\sigma^{\text{H-FLAG}}$	*rpoH* from *N. meningitidis* S4 fused to a 3' FLAG tag at *iga-trpB* intergenic locus. Erythromycin resistant	This study
S4P$_{\text{lac}}\sigma^{\text{H}}$	*rpoH* gene from *N. meningitidis* S4 at the iga-*trpB* intergenic locus. Erythromycin resistant	This study
S4$\sigma^{\text{H-FLAG}}$	*N. meningitidis rpoH* fused to a 3' FLAG tag at the endogenous *rpoH* locus. Erythromycin resistant	This study
S4$\sigma^{\text{N-His}}$	*N. meningitidis S4 rpoN* fused to a 3' His (HHHHHH) tag at the endogenous locus. Kanamycin resistant	This study
S4*ery*	Erythromycin cassette at *iga-trpB* intergenic locus. Erythromycin resistant	This study

2.2.2 Primers used in the study

For primers used for strain construction see Appendix 8.1

2.3 Genetic techniques

2.3.1 DNA extraction and product purification

For genomic DNA (gDNA) extraction from *N. meningitidis,* approximately half a loop of bacteria (10 µl) were collected from a plate and resuspended in 600 µl Nuclei Lysis solution (Wizard Genomic DNA Purification Kit, Promega) and incubated for 5 min at 80°C. A 200 µl aliquot of Protein precipitation solution (Wizard Genomic DNA Purification Kit, Promega) was added and the suspension was mixed by vortexing. Following incubation at 4°C for at least 5 min, the suspension was centrifuged 14,000 x *g* to separate the supernatant containing DNA from the precipitated material. DNA was precipitated with isopropanol and washed with 70% ethanol. Finally, the DNA pellet was resuspended in 100 µl $_{dd}H_20$.

DNA extraction from agarose gels was performed using the Wizard PCR Clean up system (Promega) according to manufacturer's directions. Briefly, following electrophoresis DNA was excised from the gel, and Membrane binding solution was added to dissolve the gel at 60°C. The mixture was transferred to a column and washed with wash solution. Finally, DNA was eluted from the column with Nuclease free water.

Plasmid purification was performed using the Gene Elute Plasmid Mini Prep Kit (Sigma-Aldrich). Briefly, 5 ml of *E. coli* culture grown overnight at 37°C was centrifuged 12,000 x *g* to pellet bacteria which were resuspended in Resuspension solution and then lysed with the lysis solution. Cell debris were precipitated by addition of neutralisation/ binding solution and the soluble fraction was loaded onto the column. The column was washed, and DNA was eluted in elution Buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2.3.2 Agarose gel electrophoresis

Agarose gels were made using 1% (w/v) Agarose (Invitrogen) dissolved in 1xTAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and 1 x SYBR Safe DNA dye (ThermoFisher). Samples were mixed with loading dye (30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF) prior to loading in the gels. HyperLadder 1kb (Bioline) was used as a size reference, and samples were separated by electrophoresis at 120V. DNA was visualised using GelDoc (Biorad gel Doc XR+).

2.3.3 Restriction enzyme digest

Plasmid DNA was digested using restriction enzymes (New England Biolabs). In brief, 3-5 µg of DNA was digested using a restriction enzyme in a 50 µl volume with Cutsmart buffer. Digestions were performed by incubation in a PCR Mastercycler pro thermocycler (Eppendorf) for at least 1 hr at 37°C, and then for 10 min at 80°C. Digested product were purified by gel extraction.

2.3.4 Transformation

Plasmid DNA (100 ng) was used to transform 50 µl of chemically competent *E. coli*. Bacteria and plasmid were mixed and left on ice for 30 min, then incubated at 42°C for 45 seconds, followed by 2 min on ice. LB was added to the bacterial suspension and the cells were incubated for 1-2 hrs at 37°C with shaking at 180 rpm. After incubation the bacteria were pelleted and resuspended in 100 µl of LB, then plated onto LB agar containing appropriate antibiotics. For electroporation, 5 µl of plasmid (concentration 100-500 ng/µl) was used to transform 50 µl of bacteria using protocols described by (Woodall, 2003).

Bacteria mixed with plasmid were incubated on ice for 30 min, then transferred to a 1 mm electroporation cuvette. The following conditions were used for electroporation using a BioRad Gene pulser: 2.5 µV, 25 µF, and 200 Ω. After electroporation 900 µl of pre-warmed LB was added and bacteria were transferred into eppendorf tubes and incubated at 37°C for 2 hrs with shaking at 180 rpm. Bacteria were pelleted by centrifugation at 12,000 x g, resuspended in 100 µl LB and plated onto LB agar containing appropriate antibiotics.

N. meningitidis was grown overnight on BHI agar, and approximately half a loop (10 µl) of bacteria was resupendend in 400 µl PBS; 10 µl of the mix was spotted onto BHI agar plates and left to dry. Next 10 µl of DNA in PBS was added to the dried spot and plates were incubated 3-5 hrs at 37°C in CO_2 5%. After incubation, bacteria from the spot were collected using a loop, and spread onto BHI agar containing antibiotics and incubated overnight at 37°C in the presence of 5% CO_2.

2.3.5 Gibson assembly

NEBuilder (New England Biolabs) website was used to design primers to amplify relevant DNA fragments by PCR. Amplified fragments containing 5' extensions were mixed with Gibson assembly mastermix containing DNA ligase, DNA polymerase and 5' exonuclease T5. Gibson reaction consists of the following steps: 1) the 5' exonuclease cleaves the 5' ends of the fragments 2) the exposed end sequences of the fragments anneal based on the complementary sequences, 3) DNA polymerase synthesises the complementary regions within the gaps on the annealed regions, 4) DNA ligase ligates the assembled fragments. The resulting assemblies are used to transform *E. coli*.

2.3.6 Construction of *N. meningitidis* mutants with σ factor deletions and tagged σ factors at the endogenous locus

Mutants lacking σ factor were constructed by gene replacement. First, pGEMT-Easy-Δ*rpoN* was constructed by amplifying DNA fragments corresponding to 479 nt upstream and 712 nt downstream of the *N. meningitidis* S4 *rpoN* ORF by PCR (See Appendix 8.1 for primers). The resulting fragments were cloned into pGEMT-Easy, flanking a kanamycin resistance cassette which was amplified from pUC19-kan. pGEMT-Easy-Δ*rpoN* was digested with BamHI and NotI, and the Δ*rpoN* fragment was purified by gel extraction prior to transformation of S4. pUC19-Δ*rpoE* was generated by amplifying 483 nt and 459 nt fragments corresponding to regions upstream and downstream of the *N. meningitidis rpoE* respectively. The fragments were cloned into pUC19 flanking a kanamycin resistance cassette using NEB Builder HiFi DNA Assembly kit (New England Biolabs). The construct was amplified from pUC19-Δ*rpoE* by PCR for transformation into S4.

Kanamycin resistant colonies were analysed by PCR. gDNA from PCR-positive clones was backcrossed into the parental strain. Backcrossed colonies were pooled (10-20 colonies) to generate S4Δ*rpoN* or S4Δ*rpoE*. gDNA from the pooled stock was checked by PCR and DNA sequencing (SourceBioscience). Attempts to make S4Δ*rpoH* and S4Δ*rpoD* were unsuccessful in line with previous reports suggesting that these genes are essential in *N. meningitidis* (Capel *et al.*, 2016).

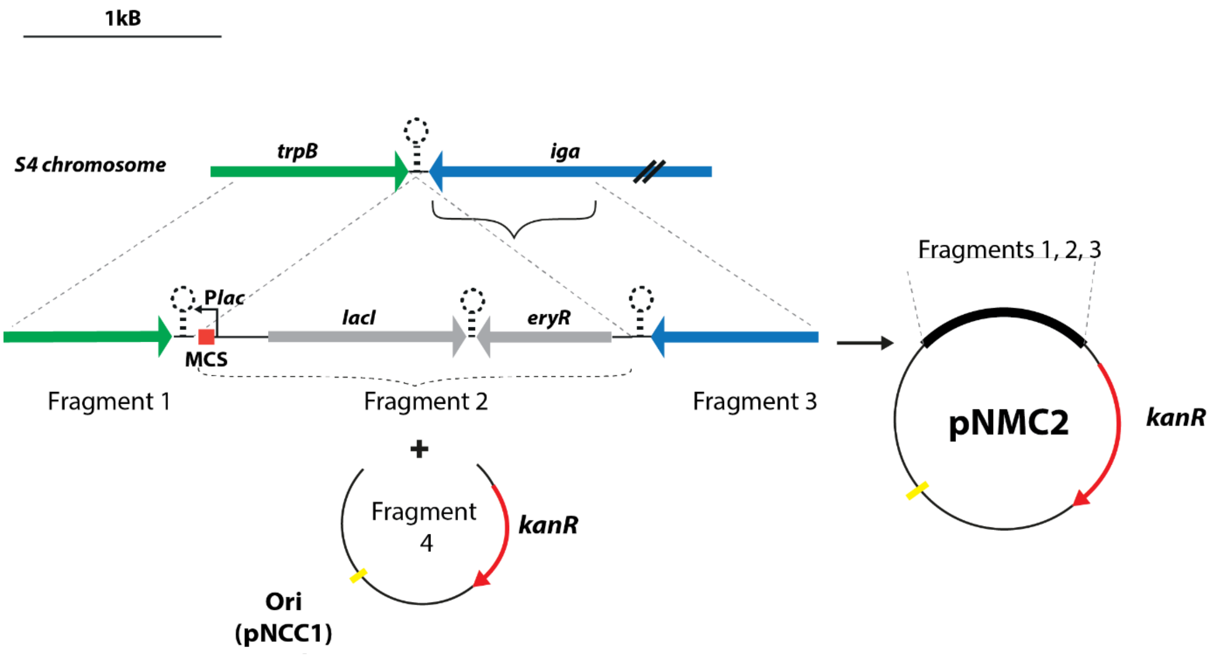
S4 harbouring C-terminal His-tagged copy of *rpoN* at the native locus was constructed as follows. Three fragments were amplified from S4 gDNA (for primers, see Appendix 8.1):

1) fragment 1 corresponding to 833 bp of the 3' of *rpoN* 2) fragment 2 corresponding to 745 bp downstream of *rpoN* stop codon, 3) fragment 4 contained 634 bp which were positioned 1728 bp away from the *rpoN*. Fragment 3 containing kanamycin resistance cassette was amplified from pUC19-kan. Four fragments were cloned using overlap PCR (Heckman *et al.*, 2007) and ligated into pGEM-TEasy. The construct was amplified from the resulting vector (pGEMT-Easy-*rpoN*-His), purified by gel extraction and used for transformation of S4. S4 harbouring C-terminal FLAG-tagged copy of *rpoH* at the native locus was constructed using four fragment Gibson Assembly (primers: Appendix 8.1): 1) fragment 1 corresponded to 322 bp of 3' of *rpoH* amplified from S4 gDNA, 2) fragment 2 amplified from pNCC1 contained erythromycin resistance cassette, 3) fragment 3 corresponded to 153 bp downstream of *rpoH* stop codon amplified from S4 gDNA, and 4) fragment 4 which was a HindIII cut pUC19. The resulting plasmid pUC19-*rpoH*$^{\mathrm{FLAG}}$ was used as a template for PCR amplification of the assembled construct and subsequent transformation in S4.

2.3.7 *N. meningitidis* strains overexpressing σ factors

Strains overexpressing σ factors were constructed by inserting the ORF of the respective σ factor at the *iga-trpB* intergenic locus of strain S4 as described previously (Ramsey *et al.*, 2012). For this purpose, pNMC2 was generated using a four fragment Gibson assembly (primers: Appendix 8.1): a) fragment 1 corresponding to 963 bp of the 3' end of *trpB* and 168 bp immediately downstream of *trpB*, b) fragment 2 corresponding to 875 bp of 3' end of *iga* and 74 bp immediately downstream of *iga*, c) fragment 3 amplified from pNCC1 vector (Wörmann *et al.*, 2016), containing a multiple cloning site (MCS), *lac* regulatory elements and erythromycin resistance cassette, and c) fragment 4 from pNCC1 with its origin of replication and a kanamycin selection marker (Figure 2.1). Genes of interest were inserted in pNMC2 at the MCS and are thus under the control of IPTG-inducible promoter. All the genes inserted in pNMC2 contained a ribosomal binding site (RBS), to ensure translation for all inserted genes. pNMC2 carrying constructs integrate into the chromosome by homologous recombination at the *trpB* / *iga* locus and transformants were selected by resistance to erythromycin.

Figure 2.1 Construction of pNMC2



For complementation with IPTG-inducible σ factor expression, genes encoding σ factors were amplified as follows: a) *rpoN* (S4 homolog of FAM18 NMC_RS01150) from either *N. meningitidis* S4 or *N. elongata* 29315 (NELON_RS02880) were amplified from gDNA (for primers, see Appendix 8.1); b) *rpoH* (S4 homolog of FAM18 NMC_RS03540) or *rpoE* (S4 homolog of FAM18 NMC_RS11230) were amplified from S4 gDNA (for primers, see Appendix 8.1). Wild-type or C terminal FLAG-tagged σ factor encoding genes were ligated into HindIII digested pNMC2 using NEB Builder HiFi DNA Assembly kit to generate pNMC2-*rpoN*^{Nel}, *rpoN*^{Nm}, *rpoE*^{Nm}, and *rpoH*^{Nm}, and pNMC2-*rpoN*$^{Nel\text{-FLAG}}$, *rpoN*$^{Nm\text{-FLAG}}$, *rpoE*$^{Nm\text{-FLAG}}$, and *rpoH*$^{Nm\text{-FLAG}}$. Plasmids were linearised by digestion with NcoI, and the products were used to transform of S4 to obtain the following strains: S4P_{lac}*Nm* σ^{N} and S4P_{lac}*Nm* $\sigma^{N\text{-FLAG}}$; S4P_{lac}*Nel* σ^{N} and S4P_{lac}*Nel* $\sigma^{N\text{-FLAG}}$; S4$P_{lac}\sigma^{E}$ and S4$P_{lac}\sigma^{E\text{-FLAG}}$; S4$P_{lac}\sigma^{H}$ and S4$P_{lac}\sigma^{H\text{-FLAG}}$. Empty pNMC2 was digested with NcoI and used to transform *N. meningitidis* S4 to generate S4*ery* strain as a control. Single erythromycin resistant colonies were analysed by PCR, and gDNA from PCR-positive colonies was backcrossed into the parental strain. Backcrossed colonies were pooled and gDNA from the pooled stock was checked by PCR and sequencing of the region of interest.

2.3.8 *N. meningitidis* strains containing *lacZ* PilE promoter fusion

To generate S4Δ*rpoN*P*pilE-lacZ*, first *rpoN* was replaced with an erythromycin resistance cassette. Briefly, regions upstream (485 bp) and downstream (712 bp) of *rpoN* were amplified from S4 (primers: Appendix 8.1). Overlap PCR was used to clone these fragments 5' and 3' of an erythromycin resistance cassette amplified from pNCC1. The sequence was ligated into pCR2.1-TOPO resulting in pCR2.1-TOPO-Δ*rpoN* which was used as a template to amplify DNA for transformation of S4. Single colonies were screened by PCR, and gDNA was isolated from PCR-positive clones for backcrossing into the parental strain. Backcrossed colonies (10-20) were pooled to generate S4Δ*rpoN::Ery*. Subsequently, the *pilE* ORF was replaced with the *lacZ* ORF, as follows: the regions immediately 5' of the *pilE* CDS were amplified from S4 gDNA (primers: Appendix 8.1). Then the region immediately 3' to the *pilE* CDS along with the kanamycin resistance cassette using S4*pilE*::*kanR* as a template (R. Exley unpublished) was amplified (primers: Appendix 8.1). The three fragments generated were joined by fusion PCR and the resulting product was ligated into pCR2.1-TOPO. The resulting plasmid pCR2.1-TOPO-Δ*pilE::*P*pilE-lacZ* was used as a template to amplify DNA for transformation of wild-type S4 or S4Δ*rpoN::Ery* to generate S4Δ*pilE*::P*pilE*-lacZ and S4Δ*rpoN*Δ*pilE*::P*pilE*-lacZ, respectively.

2.3.9 Construction of *E. coli* mutants

Markerless MG1655Δ*lacZ*Δ*rpoN* was constructed by homologous recombination in two steps (Simon *et al.*, 1983). For markerless deletion of *rpoN*, donor strain SM10 (Simon *et al.*, 1983) containing a conjugation vector pCONJ4s harbouring an *rpoN* deletion construct, and the recipient, *E. coli* MG1655 containing pKO3, were grown overnight in LB with appropriate antibiotics. For markerless *lacZ* ORF deletion, the donor strain carrying pCONJ4s with a *lacZ* deletion and recipient MG1655Δ*rpoN* containing pKO3 (Link *et al.*, 1997) were grown overnight in 5 ml LB supplemented with appropriate antibiotics. A 1 ml aliquot of each culture was centrifuged for 5 min at 13,000 x *g* and the pellets were washed three times with LB and resuspended in 1 ml fresh media. 50 μl of donor and 50 μl of recipient strains were mixed, and plated on LB and incubated for 3-5 hrs at 30°C. Bacteria were collected from the plate and resuspended in 1 ml LB. 100 μl of the suspension was then plated onto selective media supplemented with 0.02% glutamine, and plates were incubated overnight. Colonies were checked using a Safeblue Luminator for the presence of GFP as an indication of pCONJ4S integration into the MG1655 chromosome. Colonies that appeared green on the plate grown in 5 ml LB containing 0.02% glutamine, and antibiotics for 5 hrs at 30°C. An aliquot was washed three times in LB and grown overnight at 37°C with shaking at 180 rpm in fresh media. A 100 μl sample culture was plated onto salt-free 2% sucrose media, containing 0.02% glutamine to select against bacteria with pCONJ4s. Colonies were checked by PCR and sequencing.

2.3.10 *E. coli* strains containing pBAD33 and pRS415 vectors

To generate MG1655Δ*lacZ*Δ*rpoN or* MG1655Δ*lacZ* containing two vector system, the following two plasmids were transformed by electroporation: a) pBAD33 harbouring arabinose-inducible *rpoN* genes from either *E. coli* MG1655 or *N. meningitidis* S4 and b) pRS415 *lacZ* reporter harbouring *pspA* promoter sequence from *E. coli* MG1655. Briefly, *E. coli* codon optimised *rpoN* genes from either *E. coli* MG1655 or *N. meningitidis* S4 were purchased from GeneArt (Invitrogen), amplified (primers: see Appendix 8.1) and ligated into XbaI and HindIII digested pBAD33 using T4 ligase to generate: pBAD33-*rpoN*$^{E.coli}$ and pBAD33-*rpoN*^{Nm}. Then upstream region of *E. coli* MG1655 *pspA* (170 bp) was amplified (primers: see Appendix 8.1) and the fragment was cloned into SmaI/BamHI-digested pRS415 to generate pRS415-P*pspA*.

MG1655Δ*lacZ*Δ*rpoN* strains containing pBAD33 vector harbouring C-terminal HA-tagged *rpoN* genes from either *E. coli* MG1655 or *N. meningitidis* S4 were constructed as follows. Codon optimised *rpoN* genes from either *E. coli* MG1655 or *N. meningitidis* S4 were amplified from pBAD33-*rpoN*$^{E.coli}$ and pBAD33-*rpoN*^{Nm} (primers: see Appendix 8.1) and ligated into either XbaI/SphI cut pBAD33 to generate pBAD33-*rpoN*$^{E.coli}$-HA or into XbaI/HinII cut pBAD33 to generate pBAD33-*rpoN*^{Nm}-HA.

2.4 Bioinformatic analysis

The genome sequences of *N. meningitidis* isolates were obtained from the publicly available *Neisseria* genome database PubMLST (http://pubmlst.org/neisseria/) (Jolley *et al.*, 2010). BLASTn was used to identify genes of interest using full length coding sequences as the query sequence. Nucleotide or protein sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Promoter annotation was done manually based on promoter sequences reported previously (Hook-Barnard *et al.*, 2007). Percent identity was determined using percent identity matrix within CLUSTALW (Chenna *et al.*, 2003). SnapGene was used to construct plasmid maps and for automatic annotation of features and ORFs in the whole genome sequences.

2.5 β-galactosidase assays

Bacteria were grown overnight at 37°C in LB liquid with appropriate antibiotics and shaking at 180 rpm, then the overnight culture was diluted in 3 ml of LB to an OD_{600} of 0.1 in 24 deep well plates (Corning). Cultures were grown for 2.5 hrs to $OD_{600} \approx 0.5$ and protein expression was induced by addition of arabinose (final concentration 0.02%). Cultures were incubated for further 2 hrs at 37°C, and OD_{600} readings were taken to monitor growth. Alternatively, overnight cultures were diluted 1 in 5 in 1 ml of LB in eppendorf tubes and incubated for 2 hrs at 37°C, with shaking at 180 rpm. An aliquot (100 µl) of bacteria was added to a 96 well plate containing 10 µl of Popculture (Novagen) and 1 µl R-lysozyme (Novagen). Lysed cells (15 µl) were then added to a well containing 135 µl of Z-buffer (60 mM Na_2HPO_4, 40mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ + 0.27 % β-mercaptoethanol). ONPG (30 µl of 4mg/ml stock) was added to start the reaction, which was stopped by addition of 50 µl of 1 M Na_2CO_3 and the OD_{420nm} and OD_{550nm} were measured. β-galactosidase activity was expressed in Miller units (J. H. Miller, 1972), which were calculated using the following formula as described in (Griffith *et al.*, 2002): Miller Units = $(OD_{420}\text{-}1.75\ OD_{550})$ x $1000/OD_{600}$ x time x volume.

2.6 Western blot analysis

N. meningitidis whole cell extracts were prepared from bacteria grown overnight at 37°C with 5% CO_2 on solid media with appropriate antibiotics and with or without 1 mM IPTG. *E. coli* cell extracts were prepared from liquid cultures both before and after the addition of arabinose. The sample concentration was normalised to an OD_{600} of 1 in 1 ml BHI or LB, which was then centrifuged for 5 min at 12,000 x *g* and bacterial pellets were re-suspended in equal volumes of sterile water and 2X SDS-PAGE lysis buffer (1 M Tris-HCl pH 6.8 10% SDS, 30% Glycerol, 1% Bromophonol blue) containing 200 mM β-mercaptoethanol. Samples were boiled for 10 min prior to electrophoresis. Proteins were separated on 12% polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-C Extra, Amersham or Immobilon-P PVDF, Millipore) using semi-dry transfer. Membranes were blocked for 1 hr at room temperature or overnight at 4°C with in PBS+5% milk+0.1% Tween. Western blotting was performed using anti-FLAG antibody (F1804, Sigma) 1:5,000 and a goat-anti-mouse IgG HRP (final dilution, 1:10,000 DAKO), or anti-pilin (EP11270 antipeptide, 1:10,000) and a goat-anti-rabbit IgG HRP (final dilution 1:10,000 Santa-Cruz). For His-tagged constructs, anti-His antibody (1:1000, AB-18184 Abcam) and a goat-anti-mouse IgG HRP (final dilution 1:10,000, DAKO) were used. For HA-tagged constructs, anti-HA antibody (1:1000, Cell Signaling Technology) and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) were used. Anti-RecA (1:5,000 ab63797, Abcam) followed by a goat-anti-rabbit IgG HRP (final dilution 1:10,000, Santa-Cruz) or Coomassie staining were used as loading controls. All antibodies were incubated for 1 hr at room temperature in PBS+5% milk+0.1% Tween and washed three times with PBS+0.1% Tween. Bands were visualised using ECL detection reagent (GE Healthcare).

2.7 RNA isolation

N. meningitidis was grown at 37°C in liquid BHI and at relevant times a volume equivalent to $OD_{600} \approx 8$ was centrifuged at 4,000 rpm for 10 min. The supernatant was removed and total RNA was isolated from pellets using TRIzol extraction (ThermoFisher). Briefly, cell pellets were re-suspended in 1 ml of TRIzol and incubated at room temperature for 5 min before adding 200 µl of chloroform. Samples were shaken by hand for 15 s, incubated at room temperature for 2-3 min, then centrifuged at 12,000 x *g* for 15 min at 4°C, and the aqueous phase was transferred to a fresh tube. RNA was precipitated by adding 500 µl isopropanol, followed by centrifugation at 20,000 x *g* for 30 min at 4°C. RNA pellets were washed with 75% ethanol then air-dried and re-suspended in diethylpyrocarbonate (DEPC) treated-water. Following DNase treatment for 2 hrs at 37°C, RNA was extracted with phenol/chloroform (5:1). The aqueous phase was re-extracted with DEPC treated water and chloroform/isoamyl alcohol. Samples were vortexed and centrifuged at 20,000 x *g* for 5 min at 4°C. The aqueous phase was transferred to a tube containing 33 µl of 3M NaAc (pH 4.5) and 812.5 µl of 99.5% ethanol, and RNA precipitated over 2 hrs or overnight at -20°C. Following centrifugation at 20,000 x *g* for 30 min at 4°C, RNA pellets were washed with 75% ethanol and centrifuged again 10 min at 20,000 x *g* then air dried, and re-suspended in 200 µl DEPC water.

2.8 Quantitative RT-PCR (qRT-PCR)

RNA (2.5 µg) was used to synthesise cDNA with primers specific for the target gene (Appendix 8.1) containing a tag sequence (CCG TCT AGC TCT CTC TAA TCG) which is not present in *N. meningitidis* genome. Briefly, RNA was reverse transcribed using Reverse

Transcriptase III polymerase (Invitrogen) and treated with RNase H for 20 min at 37°C. cDNA was purified using Wizard Gel PCR Purification kit (Promega). qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems). Primer efficiency was evaluated using serial dilutions of gDNA to generate a standard curve. The slope of the standard curve for each primer pair was calculated. StepONEPlus Real time PCR software was used to collect qRT-PCR data; the ΔCt method (where the Ct is the threshold cycle) was used to analyse the data (Schmittgen 2008). Results are presented as the normalised R value (where R value is calculated as 2^{-Ct}) of three biological replicates; tmRNA was detected with primers (Appendix 8.1) and used as a control.

Table 2.4 Oligonucleotides used for qRT-PCR

Oligo-nucleotide	**Sequence**	**Description**
M64	CGTCCGTATGTATATGCGC	F for *rpoD*
M65	CCGTCTAGCTCTCTCTAATCGAGATTTCCGCAATGGATCC	cDNA synthesis *rpoD*
M66	GCGTAAACTGCTTCTGCAT	F for *rpoE*
M67	CCGTCTAGCTCTCTCATGGCAAAAAGCCAGCTG	cDNA synthesis *rpoE*
M68	TTTGAGAAACTGGCGTTTGG	F for *rpoH*
M69	CCGTCTAGCTCTCTCTAATCG ACTTCGGAAAGTTTGACACC	cDNA synthesis for *rpoH*
M70	CCGTCTAGCTCTCTCTAATCG ATGCTGTCGAGGGCGTTTCG	cDNA synthesis for *rpoN*
M71	CAAGCCCTGACTGCATTGC	F for *rpoN*
M72	CCGTCTAGCTCTCTCTAATCG CCTTCAACCTTAACCGATGC	cDNA synthesis for *pilE*
M73	AAGGTCAAAAATCCGCAGTG	F for *pilE*
M74	CCGTCTAGCTCTCTCTAATCG	cDNA synthesis for tmRNA

	AACCCGGTAGGAAACCAGTC	
M75	AGTCGCAAACGACGAAACTT	F for tmRNA
M76	CCGTCTAGCTCTCTCTAATCG	qRT-PCR tag

2.9 Primer extension

Primer extension was carried out using 1 μg of RNA isolated from *N. meningitidis* grown to mid-log phase ($OD_{600}\approx$ 0.4-0.5). Primers (P1 and P2, Appendix 8.1) were radiolabelled using [γ-^{32}P]ATP (PerkinElmer). A mix of radiolabelled primers and RNA were incubated for 3 min at 95^{o}C and rapidly cooled on ice for 4 min. Reverse transcription was performed using Superscript II (Invitrogen) by incubating samples at 45^{o}C for 60 min, then stopped by deactivating the enzyme for 10 min at 70^{o}C. Formamide loading dye (10 μl) was added to each reaction and samples were heated briefly at 95^{o}C. 15 μl of each sample was loaded on 12% denaturing polyacrylamide sequencing gel, and electrophoresis was performed for 2-3 hrs 20-25W until bromophenol blue dye reached the bottom of the gel. Products were analysed using phosphoimager (FujiFilm).

Table 2.5 Oligonucleotides used for primer extension

Oligonucleotide	Sequence	Description
P1	CCTTCTTACCAGCAAGCGGC	P1 Primer extension
P2	CGTAATGTATTTGACCGTGG	P2 Primer extension
C	ATACGGATATTGTGCGGTGC	C Primer extension (tmRNA)

2.10 Northern blot analysis

RNA (20 µg) was precipitated overnight in 3M NaAc (pH 5.2) and ethanol. After overnight precipitation, RNA was pelleted by centrifugation at 20,000 x *g* for 30 min and resuspended in 15 µl sample buffer (40 mM HEPES, pH 7.4, 2 mM EDTA, pH 8.0, 4 M formaldehyde, 60% formamide). RNA was denatured at 65°C for 3 min, and loading buffer (0.1% w/v xylene cyanol, 0.1% w/v bromophenol blue, 0.1% w/v orange G, 10 mM EDTA, 95% deionized formamide) was added before loading on 1.5% agarose formaldehyde gels in HEPES buffer. RNA was transferred to Hybond-N membrane (GE Healthcare) overnight in SSC buffer (Sambrook and Russel, 2006), then crosslinked to the membrane using a UV crosslinker (Stratagene; 1500 x 100 µJ/cm^2). The membrane was pre-hybridised in Amersham Rapid-hybridisation buffer (GE Healthcare) for 2 hrs at 55°C. PCR fragments complementary to the RNA transcript of interest were used as a probe, and radiolabelled with [γ-^{32}P]ATP (PerkinElmer). Radiolabelled probes were added to the membrane in hybridisation buffer and incubated overnight at 65°C. Prior to detection the membrane was washed with 2 x SSC, 0.1% SDS and subsequently with 0.5 x SSC, 0.1% SDS for 20 min at 65°C. The membrane was exposed to Phosphor Screen and signals were detected using a

Fuji FLA-500 laser scanner. An AIDA image analyser was used to quantify the signal intensity.

Table 2.6 Oligonucleotides used for Northern blotting

Oligo-nucleoide	Sequence	Description
M60	ACTCGACCACTGCGGATTTTTGACCTTCTGCCAAAGTCAGGGCTTCGGAC	*pilE* Northern Blotting probe
M61	CACAGGCGGATTCTTGGTCGGAAAGCGGGTGTTCGCATACTTGCGCGTGC	*rpoN* Northern Blotting probe
M62	AACCCGGTAGGAAACCAGTC	R for tmRNA Northern Blotting probe
M63	AGTCGCAAACGACGAAACTT	F for tmRNA Northern Blotting probe

2.11 Differential gene expression analysis by RNA-sequencing

RNA was prepared from S4$P_{lac}\sigma^{H\text{-}FLAG}$ and S4*ery* grown on three independent occasions. Cultures were incubated with shaking at 180 rpm at 37°C until $OD_{600} \approx 0.4$-0.5 and IPTG (final concentration 1mM) was added. Cultures were incubated for an additional 10 min, after which bacterial pellets were collected by centrifugation. Purified RNA was checked for degradation and purity using TapeStation (Agilent) and 20 ng of RNA from three biological repeats was sent to Vertis Biotechnologies for RNA-sequencing. Vertis Biotechnologies performed the sequencing, mapping to reference genomes and initial differential gene expression analysis as follows: first, ribosomal RNA was removed from the total RNA using RiboZero rRNA Removal Kit (Epicentre). Ribodepleted RNA was fragmented and adapter was ligated to the 3' end of the RNA. cDNA was synthesised using 3' adapter primer and M-MLV reverse transcriptase, then purified and 5 Illumina TruSeq sequencing adapter was added. The products were amplified by PCR before purification with Agencourt AMPure XP kit (Beckman Coulter Genomics). TruSeq sequencing adapters contain TruSeq barcode sequences which are part of the 5' and 3' TruSeq sequencing adapters. The cDNA pool was size selected and sequenced on Illumina NextSeq 500 system using 75 bp read length. The reads were aligned to *N. meningitidis* 8013 (NC_008767) and FAM18 (NC_017501) reference genomes and the quality of the data was assessed using FastQC. Differential gene expression analysis was performed using DESeq2 (Love *et al.*, 2014) and $\log_2$ was used to represent the fold change in gene expression ($\log_2$ Fold change).

2.12 Statistical analysis

Statistical analyses of results from qRT-PCR was performed using either one-way ANOVA (if single strain/condition was used) or two-way ANOVA and Tukey's (if multiple strains/conditions were used). Results from β-galactosidase assays were similarly analysed using two-way ANOVA or using Student's t-test (when two groups were compared).

3 Results: Bioinformatic and functional analysis of class II *pilE* promoters

The transcriptional activity of a gene is primarily determined by the sequence composition of its upstream region, which consists of a) promoters that are recognized by different σ factors, and additional upstream sequences that can interact with trans-acting factors that modulate transcription. Bacterial promoters can be divided into two groups that differ in sequence and in their ability to bind various σ factors: group 1 is comprised of σ^{70}-dependent or -10 (TATAAT) and -35 (TTGACA) promoter elements, and group 2 consists of σ^{N}-dependent promoters that are characterised by -12 (TTGC) and -24 (YTGGCACG) recognition motifs (Feklístov *et al.*, 2014). In addition to the sequence of the consensus elements, the spacing between each element within the promoter group plays an important role in promoter activity (Hawley & McClure, 1983). σ^{70} -dependent promoter analysis across bacterial species and atomic structures of RNAP-σ^{70} complex bound to the promoters have shown that the -10 and -35 elements are 17+1 nucleotides (nt) apart. In contrast, group 2 promoter alignments demonstrated that there are 7 nt between the -12 and -24 promoter elements. Group 1 promoters can be recognized by multiple σ^{70} family members with different efficiencies; housekeeping σ^{D} has a strong preference for consensus -35 sequences whereas alternative σ^{70} members modulate their promoter specificity by tolerating only partially conserved -35 sequence (Paget, 2015). Group 2 promoters require activators (bEBP) in addition to σ^{N}. Furthermore, conserved -12/-24 sequences are essential for optimal promoter function.

In this chapter the upstream region of class II *pilE* in *N. meningitidis* has been investigated. Specifically we aimed to determine the conservation of promoter sequences and identify factors that could play a role in class II *pilE* transcription modulation.

3.1 Comparison of *pilE* promoter sequences among *N. meningitidis* isolates

Pilin gene transcription has been studied in *N. gonorrhoeae* which has class I *pilE*. Upstream of gonococcal *pilE* there are two putative promoter elements, σ^{70}- and σ^{N}-dependent motifs, that share high sequence identity with the consensus bacterial promoters (Fyfe *et al.*, 1993; Laskos *et al.*, 1998). In addition, comparison of *N. gonorrhoeae* and *N. meningitidis* class I *pilE* loci has revealed high homology between the two (Laskos *et al.*, 1998). Both gonococcal and meningococcal class I pilin promoters have similar spacing between the individual promoter elements and position relative to the *pilE* start codon (Figure 3.1A). However, sequence comparison has shown that despite the overall similarity in modular composition of the promoter, the specific sequence of promoter elements differs between *N. gonorrhoeae* and different class I *pilE* containing *N. meningitidis* isolates. For example, the class I *pilE* expressing *N. meningitidis* strains MC58 and 8013 contain identical -10 and -12 promoter elements to those found upstream of gonococcal *pilE*, whereas the -24 and -35 sequences exhibit sequence variation compared to the corresponding promoter regions in *N. gonorrhoeae*. In *N. meningitidis* 8013 the -12/-24 promoter has not been annotated as, unlike the -24 element in *N. gonorrhoeae* and MC58 *pilE* and MC58, it contains only two out of the four key nucleotides at the -24 element suggesting the function of -12/-24 promoter may vary in a strain-dependent fashion (Taha *et al.*, 1996). Interestingly, *N. gonorrhoeae* and meningococcal MC58 class I *pilE* promoters have overlapping -10/-35 and -12/-24 recognition sequences (Figure 3.1A). However, only the σ^{70}-dependent promoter is involved in *pilE* expression in class I *pilE* containing *Neisseria*, and the *pilE* transcriptional start site (TSS) is identical in both. These observations suggest that either a) the *pilE* σ^N-dependent promoter is non-functional, b) σ^N is inactive under the conditions tested, or c) activators required for σ^N-dependent transcription are not present in these strains. Indeed, σ^N has been shown to be non-functional in *N. gonorrhoeae* and class I *pilE* expressing *N. meningitidis* (Laskos *et al.*, 1998). Interestingly, despite containing an inactive σ^N, the σ^N-dependent *pilE* promoter was shown to be functional in *E. coli* which contains functional σ^{70} and σ^N. Mutagenesis analysis of gonococcal *pilE* promoter regions in *E. coli* revealed that transcription from the σ^{70}-dependent promoter decreases in the presence of the σ^N-dependent promoter possibly due to σ^N binding to the overlapping promoter (Carrick *et al.*, 1997). This has led to the hypothesis that the σ^N-dependent promoter located upstream of *pilE* in *N. gonorrhoeae* is unable to initiate transcription in *E. coli* as the appropriate activator might be absent (Carrick *et al.*, 1997). Indeed, *pilE* promoter reporter fusions have demonstrated that σ^N-dependent transcription of *N. gonorrhoeae* pilin can take place in *E. coli* providing that NifA, σ^N activator from *Klebsiella*, is present (Boyle-Vavra *et al.*, 1993), further suggesting that gonococcal σ^{70}- and σ^N -dependent *pilE* promoters are functional. σ^N-dependent pilin gene transcription has previously been described in *Pseudomonas aeruginosa* which contains *pilA*, a homologue of *pilE*. Transcription of *pilA* originates from a σ^N-dependent promoter and requires PilR, which is an activator of *P. aeruginosa* σ^N. Analysis of *N. meningitidis* class I and gonococcal *pilE* promoter activity in *P. aeruginosa* revealed that activity from the gonococcal *pilE* σ^N -dependent promoter can be detected and was dependent on the presence of *P. aeruginosa* PilR, a σ^N-dependent activator of *pilA* transcription (Carrick *et al.*, 1997; Fyfe *et al.*, 1995b).

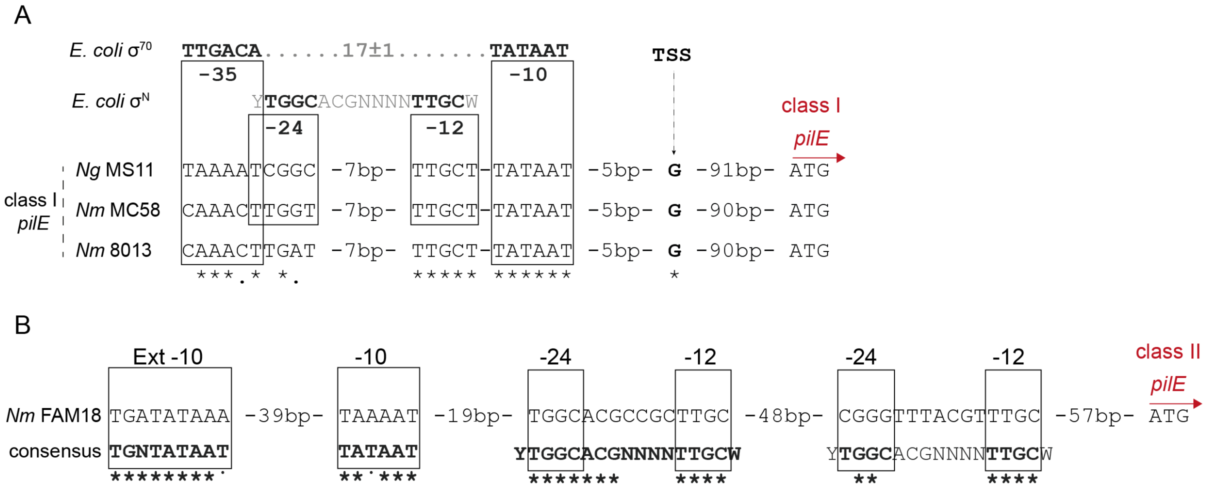


Figure 3.1 ***pilE*** **promoter annotation in pathogenic** ***Neisseria***

A. Nucleotide sequence alignment of 126 bp region upstream of the *pilE* start codon in *N. gonorrhoeae* (*Ng*) MS11 and *N. meningitidis* (*Nm*) MC58 and 8013 (class I *pilE*). Transcriptional start site (TSS) shown in bold and putative promoter elements -10/-35 and -12/-24 are boxed based on promoter annotation reported previously (Carrick *et al.*, 1997; Taha *et al.*, 1996). Consensus sequences (σ^{70} and σ^{N}) are shown in bold. B. Comparison of the putative promoters annotated upstream of *pilE* from *N. meningitidis* FAM18 (class II *pilE*) by Aho *et al.* (Aho *et al.*, 1997) with *E. coli* consensus elements (in bold). Y is a pyrimidine, W stands for A or T, and N is any nucleotide.

The sequence upstream of class II *pilE* has only been annotated in one strain of *N. meningitidis*, FAM18 (Aho *et al.*, 1997). The sequence and number of promoters as well as the relative position of the promoter sequences is distinct in class II *pilE* compared to class I *pilE* (Figure 3.1B). The class II *pilE* promoter contains a putative extended -10 motif (TGnTATAAT) which is not present in *N. gonorrhoeae* or the class I *pilE* locus. In *E. coli* an extended -10 is known to enhance the strength of a -10 promoter in the absence of a conserved -35 region (Keilty *et al.*, 1987); of note, no consensus -35 sequence was identified upstream of the extended -10 element of class II *pilE* based on the FAM18 sequence (Aho *et al.*, 1997). An additional putative -10 sequence has been annotated upstream of class II *pilE* in FAM18 similarly without an obvious cognate -35 element. This is consistent with the recent work that has shown that the σ^{70}-dependent promoters class I *pilE* expressing *N. meningitidis* 8013 genome lacks consensus -35 promoter regions (Heidrich *et al.*, 2017). Unlike class I *pilE*, the class II *pilE* promoter region harbours two putative -12/-24 sequences, although only one -12/-24 element resembles consensus σ^{N}-dependent sequence (TGGCACG-N_4-TTGC); the second -12/-24 motif (cGGg-N_7-TTGC) contains only partial homology to the consensus -24 region (two out of four consensus nucleotides) (Barrios *et al.*, 1999). In *E. coli* all four consensus -24 promoter residues play a crucial role in interacting with σ^{N} and determining the position of σ^{N} at the promoter site (Barrios *et al.*, 1999; Doucleff *et al.*, 2007), and alterations within the -24 sequence impair σ^{N} binding to promoter site (Buck, 1986; Buck *et al.*, 1985).

No overlap between putative *pilE* promoter sequences is observed in FAM18 suggesting that the transcription activation profile maybe different between class I and class II *pilE* expressing *N. meningitidis*. To understand the divergence of regulatory signals between class I and class II *pilE* and to identify possible promoter elements that could play a role in class II *pilE* expression, we first aimed to determine the presence and conservation of putative promoter regions across a range of *N. meningitidis* isolates.

As class I *pilE* promoter analysis has only been performed in two meningococcal isolates (8013 and MC58), and the class II *pilE* upstream region has been bioinformatically annotated in just a single *N. meningitidis* strain FAM18, we analysed the composition and sequence of individual *pilE* promoters in a collection of meningococcal isolates comprising 213 class I and 77 class II *pilE* sequences (Wörmann *et al.*, 2014). The collection included strains from 27 different clonal complexes isolated in 34 different countries between 1937-2011

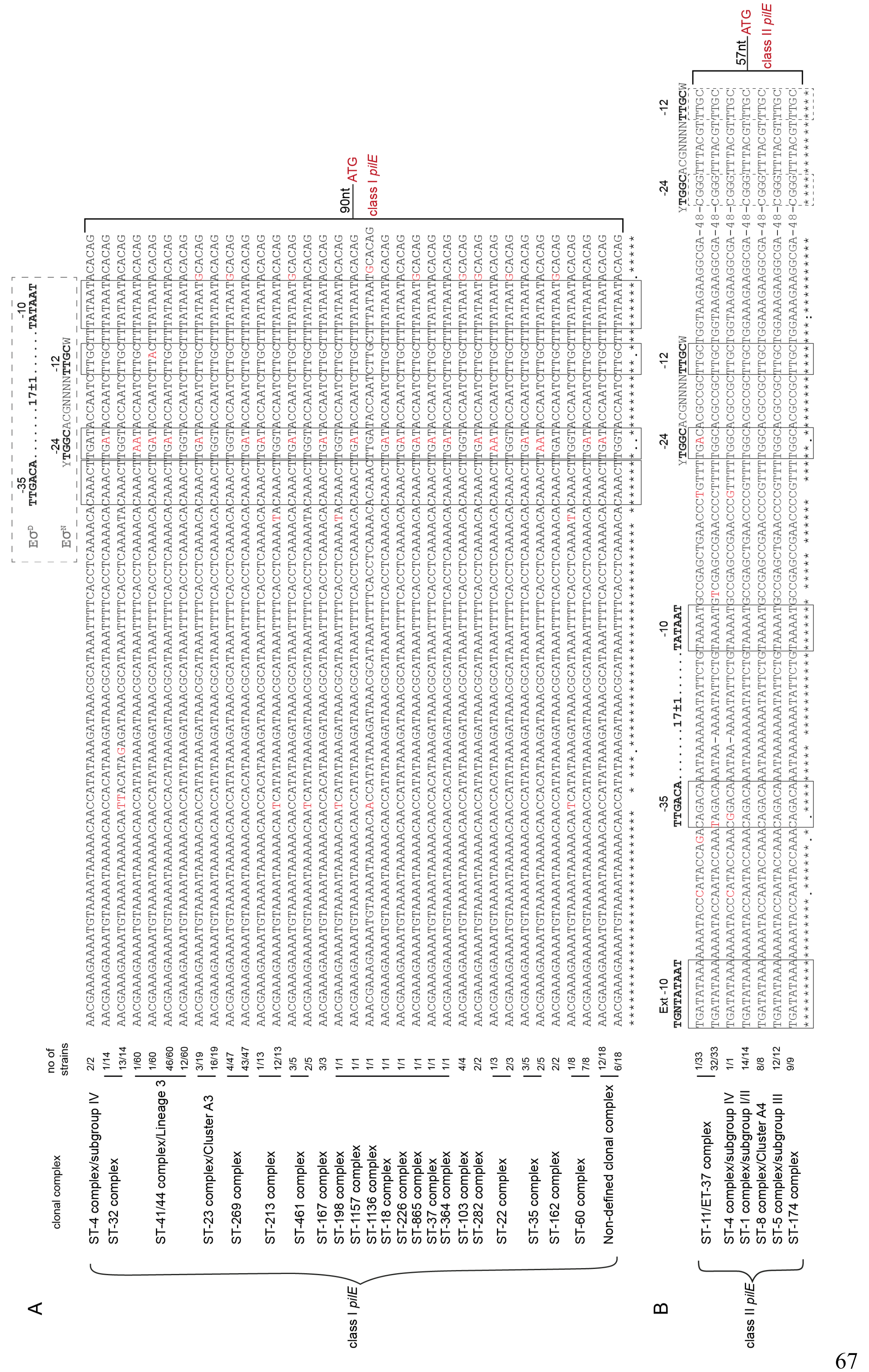
(Wörmann *et al.*, 2014). The sequences of either class I or class II *pilE* loci were extracted from the pubMLST database using full length pilin coding sequence and 500 bp of sequence flanking *pilE* from either 8013 or FAM18 as the query sequence. The nucleotide sequences of the *pilE* promoter regions and coding sequences of *pilE* were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Alignment with previously described meningococcal promoters (Aho *et al.*, 1997; Fyfe *et al.*, 1995a) and manual inspection for bacterial promoter consensus sequences (Hook-Barnard & Hinton, 2007) was used to annotate sequences corresponding to -10/-35 and -12/-24 motifs in the class I *pilE* upstream region and extended -10, -10/-35 and -12/-24 sequences in class II *pilE* region.

Analysis of class I *pilE* loci revealed that the σ^{70} and σ^{N} -dependent promoter elements reported previously were present in all 213 class I *pilE* containing isolates, and the position of the putative promoters relative to the class I *pilE* start codon was conserved among all isolates (Figure 3.2). Detailed sequence analysis of the σ^{70}-dependent promoters revealed conservation of both -10 and -35 elements across class I *pilE*-containing *N. meningitidis*; the -10 promoter was exactly matched to the consensus -10 sequence (TATAAT), whereas the putative -35 region contained only two out of six consensus nucleotides (caaACt and the consensus TTGACA), which is in line with the observation reported in *N. meningitidis* 8013 and MC58 class I *pilE* analysis (Carrick *et al.*, 1997; Heidrich *et al.*, 2017). The mis-matches in the putative -35 region demonstrated that the *N. meningitidis pilE* -35 promoter elements although conserved among *N. meningitidis* strains, do not correspond to the consensus sequence. The absence of full consensus -10/-35 promoter suggests that additional activators might be required to compensate for the lack of -35 promoter region (Heidrich *et al.*, 2017). The overlapping -12/-24 sequence also showed divergence from the consensus sequence:

138 out of 213 strains contained at least one nucleotide difference with the consensus -24 sequence, suggesting that this promoter might be inactive in these isolates, for reasons discussed earlier in this section (Figure 3.2). One isolate, belonging to the ST-41/44 complex, had an additional mutation in the -12 region, further indicating that this promoter might be dispensable for class I *pilE* expression in meningococcus. The sequence upstream of the putative -35 element was conserved in all 213 class I *pilE* containing *N. meningitidis*, with only four nucleotides differences seen across all isolates.

Figure 3.2 Alignment of nucleotide sequence upstream of *pilE* in *N. meningitidis*

A. class I *pilE* and B. class II *pilE*. Clonal complex and the number of strains with the indicated sequences is shown. Putative promoters corresponding to -10/-35 and -12/-24 elements are boxed and consensus promoter sequences are shown above the boxes in bold. Nucleotides shown in red exhibit little or no conservation within promoter element.


A
B
class I pilE
class II pilE
clonal complex
no of strains
ST-4 complex/subgroup IV
ST-32 complex
ST-41/44 complex/Lineage 3
ST-23 complex/Cluster A3
ST-269 complex
ST-213 complex
ST-461 complex
ST-167 complex
ST-198 complex
ST-1157 complex
ST-1136 complex
ST-18 complex
ST-226 complex
ST-865 complex
ST-37 complex
ST-364 complex
ST-103 complex
ST-282 complex
ST-22 complex
ST-35 complex
ST-162 complex
ST-60 complex
Non-defined clonal complex
ST-11/ET-37 complex
ST-4 complex/subgroup IV
ST-1 complex/subgroup I/II
ST-8 complex/Cluster A4
ST-5 complex/subgroup III
ST-174 complex
EσD
EσN
-35
TTGACA.......17±1......TATAAT
-10
-24
-12
YTGGCACGNNNNTTGCW
Ext -10
TGNTATAAT
90nt
57nt
ATG
class I pilE
class II pilE

In the class II *pilE* promoter region, the extended -10 sequence and -10 sequence were conserved across all 77 isolates examined and had only one mis-match compared to the *E. coli* -10 sequence (Figure 3.2). The -35 promoter motif was annotated based on the consensus -35 region in *E. coli*. Unlike the putative -35 sequence found in class I *pilE* isolates, the class II -35 promoter sequence had higher resemblance to the -35 promoter and containing four out of six conserved nucleotides (caGACA and consensus TTGACA). This suggests that -10/-35 promoter might exhibit different σ^{70} factor specificity in isolates with class II *pilE* compared to isolates with class I *pilE*. The spacing between the class II *pilE* -10 and -35 elements was variable due to the presence of a poly-A tract: 34 out 77 isolates contained poly-A_6 tract rather than poly-A_7. Class II *pilE* is not known to undergo phase variation although polymorphic tracts present in close proximity to -35 elements are known to mediate phase variation in number of genes (Browning *et al.*, 2016; Sarkari *et al.*, 1994). Putative -12/-24 sequences were conserved among all 77 isolates and were identical to the previously reported promoters in FAM18 (Aho *et al.*, 1997). Similarly to FAM18 promoter sequence, one of the -12/-24 promoters contained low homology of the -24 element (two out of four nucleotides), which is likely to negatively impact its activity based on the reported analysis of the -12/-24 promoters harbouring similar mutations in other organisms (Barrios *et al.*, 1999; Buck *et al.*, 1985; Keseler *et al.*, 1995). For instance, in *N. meningitidis* 8013 that harbours class I *pilE*, -12/-24 promoter was not annotated because -24 region contained only two out of the four consensus nucleotides (Figure 3.1) (Taha *et al.*, 1996). We therefore did not consider this promoter in the subsequent analysis. The sequence of the other -12/-24 promoter was remarkably conserved, exhibiting 100% nucleotide sequence identity to the -12/-24 consensus in 76/77 class II *pilE* containing *N. meningitidis* (Figure 3.2). This conservation and the non-overlap with the σ^{70}-dependent promoter that is seen in class I *pilE* raised possibility that -12/-24 promoter might be involved in class II *pilE* regulation. Since the -12/-24 sequence requires σ^{N} for transcription initiation, we next aimed to bioinformatically examine whether class II *pilE* expressing strains contain σ^{N}-coding gene *rpoN*.

3.2 Meningococcal isolates contain truncated *rpoN* with the majority of strains retaining the RpoN box in the predicted σ^{N} sequence

As discussed earlier in Section 1.5.1.4, σ^{N} has been reported to be non-functional in *N. gonorrhoeae* due to a frameshift mutation in the coding sequence that results in loss of the HTH motif and the RpoN Box which are involved in binding to the -12 and -24 promoter regions respectively (Laskos *et al.*, 1998; Rendon *et al.*, 2013). In addition, the failure to detect σ^{N} in *Neisseria* using a mAb raised against *S. typhimurium* RpoN (Laskos *et al.*, 1998) as well as the lack of transcriptional activity from the σ^{N}-dependent pilin promoter in *N. gonorrhoeae* provided further evidence that this σ factor is non-functional in gonococcus. This is in contrast to non-pathogenic species *N. elongata*, in which the *rpoN* gene encodes a protein with both DNA binding motifs that binds the *pilE* promoter and activates *pilE* transcription (Rendon *et al.*, 2013). In the same study, it was reported that *N. meningitidis rpoN* encodes a truncated σ^{N}, which lacks an obvious HTH motif, but retains the RpoN box (Rendon *et al.*, 2013), although this may depend on the isolate. For example, comparison of σ^{N} sequences from several *N. meningitidis* isolates demonstrated that *N. meningitidis* 8013 *rpoN* encodes a truncated σ^{N} similar to *N. gonorrhoeae*, whereas two other *N. meningitidis* isolates contained a different *rpoN* sequence that codes for a different truncated form of σ^N which lacked HTH but retained RpoN box domain (Rendon *et al.*, 2013).

σ^N domain organisation has been studied extensively in *E. coli* and the protein is divided into three functional regions: Region I, containing activator binding domain, Region II which is a variable non-essential region involved in DNA melting, and Region III implicated in interaction with RNAP, promoter recognition and binding (Figure 3.3) (Buck *et al.*, 2000). The C terminal Region III is of particular interest as it contains the DNA binding and recognition motifs: the HTH and characteristic RpoN box (ARRTVAKYRE). Interestingly mutagenesis studies in *E. coli* have demonstrated that σ^N interacts more strongly with the -24 element. Furthermore, the RpoN box plays an important role in mediating σ^N dependent promoter recognition, as mutations in the RpoN box reduce the binding of σ^N to the target promoter below 20% (Doucleff *et al.*, 2007). In contrast to RpoN box, absence of HTH domain deletion does not abolish σ^N promoter binding (Wong *et al.*, 1994).

Since there is a variation in the small number of meningococcal σ^N sequences, we first aimed to investigate the conservation of *rpoN* in the collection of 290 *N. meningitidis* isolates (described in Section 3.1, (Wörmann *et al.*, 2014). *rpoN* sequence from FAM18 was used as the query sequence to extract nucleotide sequences from meningococcal genomes in the PubMLST database. The nucleotide sequences were then translated using EMBOss TRanseq to determine deduced amino acid sequences which were then aligned using CLUSTAL Omega. In total 288 out of 290 strains were subject to detailed comparison of σ^N sequences: two isolates belonging to ST-162 were excluded as we were unable to extract complete *rpoN* sequence using whole genome sequencing (WGS) data in the pubMLST database.

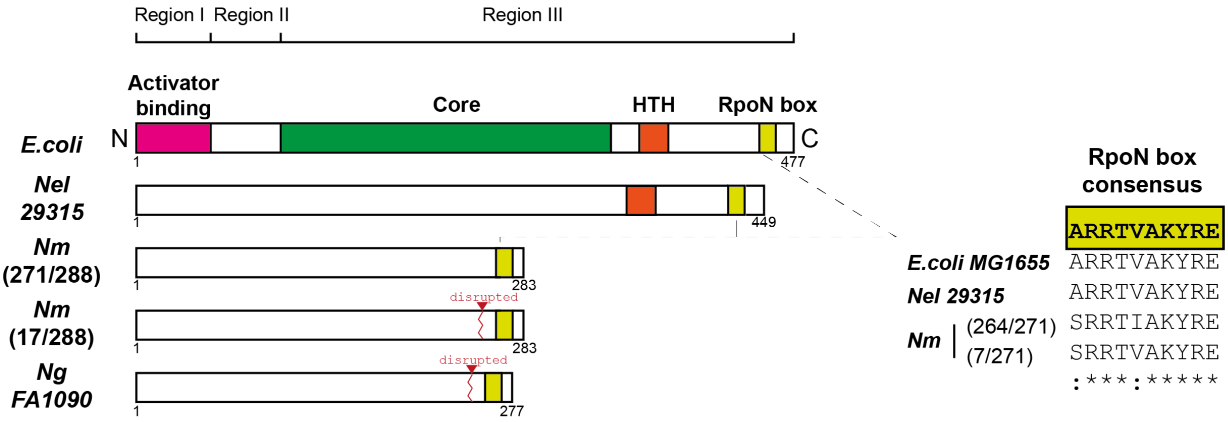


Figure 3.3 Conservation of σ^N characteristic RpoN box motif in *N. meningitidis* isolates

Schematic representation of σ^N in *E. coli* divided into three functional regions: Region I containing activator binding site, Region II and Region III from the core of σ^N and Region III also contains DNA binding motifs including HTH and RpoN box (Buck *et al.*, 2000). The schematic of the deduced σ^N in commensal *N. elongata* (*Nel* 2931; amino acids 1 to 449) and *N. gonorrhoeae* (*Ng* FA1090, amino acids 1-277) is shown together with the 288 isolates of *N. meningitidis* (*Nm*). A total of 17 *N. meningitidis* strains harbour a frameshift mutation found in *N. gonorrhoeae* strains which results in RpoN box being abolished. Amino acid sequence alignment of the conserved RpoN box of σ^N from *E. coli*, *N. elongata* and *N. meningitidis* is shown.

Bioinformatic analysis of the deduced σ^N amino acid sequences revealed no extensive variation in the meningococcal σ^N sequences, on average exhibiting >95% sequence homology (Appendix 8.2). Furthermore, we did not observe any association between a given σ^N sequence and class I or class II *pilE* promoter sequence. Some *N. meningitidis* isolates (17/288) harboured a truncated form of σ^N due to a frameshift mutation as described previously (Laskos *et al.*, 1998). The resulting premature stop codon puts the RpoN box out of frame and leaves σ^N without any putative DNA binding regions and therefore it is unlikely to be functional. However the majority of *N. meningitidis* isolates (271/288) contained a truncated σ^N which lacked HTH motif but retained the signature RpoN box (Figure 3.3). Given that in *E. coli* σ^N harbouring RpoN box but not HTH can still bind the promoter

(Taylor *et al.*, 1996), the presence of conserved RpoN box in these meningococcal isolates meant that we could not exclude the possibility that this form of σ^N could retain binding to the -24 element of the -12/-24 promoter and potentially regulate class II *pilE* promoter activity.

Our analysis shows that *N. meningitidis* σ^N shares 30% and 54% sequence identity with *E. coli* and *N. elongata* σ^N respectively and RpoN box domain is the only conserved domain among these species. In *E. coli* Region I containing the activator-binding site has been shown to interact with bEBPs whereas the core domain within Region III interacts with RNAP (Figure 3.3). Considering that *N. meningitidis* σ^N shares no homology with the *E. coli* activator-binding site and core region, it is possible that meningococcal σ^N, if functional, might have a distinct domain organisation and therefore interact with RNAP and activators differently compared to *E. coli* σ^N.

3.3 *pilE* promoter sequences are functional in *E. coli*

To begin analysis of the activity of class II *pilE* promoter, we investigated further whether the putative promoter sequences identified by bioinformatic analysis in Section 3.1 can activate transcription. The activity of each of the class II *pilE* upstream sequence harbouring putative promoter elements was therefore analysed using a heterologous *E. coli* system. This approach is preferable as it allows to investigate the activity of promoters in the presence of known functional *E. coli* σ^{70} and σ^N factors as well as the activators necessary for *E. coli* σ^N-dependent transcription. The class II *pilE* locus from *N. meningitidis* FAM18 was used as a template for amplification of the following sequences upstream of the *pilE* start codon: i) a 322 bp region comprising all putative promoters (extended -10, -10/-35 and -12/-24), ii) a 199 bp region corresponding to the -10/-35 and -12/-24 sequences, and iii) a 153 bp region corresponding to the -12/-24 promoter alone. The three fragments were cloned into pRS415 upstream of *lacZ* reporter gene to create transcriptional fusions which contained the ribosomal binding site (RBS) and 5' UTR of *lacZ* (Figure 3.4), enabling analysis of promoters using β-galactosidase activity as a reporter. The plasmids were introduced into *E. coli* DH5α and the activity of the different *pilE* upstream regions was analysed during bacterial growth at three time points: 2, 4 and 6 hours reflecting early exponential, mid logarithmic and early stationary phases of growth respectively. *E. coli* containing either no vector or pRS415 harbouring promoterless *lacZ* were used as controls. β-galactosidase assay shows that both control strains had background activity. Each reporter fusion containing different *pilE* upstream regions was active in *E. coli* throughout growth as β-galactosidase activity was significantly higher than in control strains (Figure 3.4). The activity of each *pilE* upstream sequence followed the same pattern at every time point during the growth: the highest activity was detected from the 322 bp fragment (extended -10 motif, -10/-35 and -24/-12) and from the 199 bp fragment (-10/-35 and -12/-24 elements). There was no statistical difference in β-galactosidase activity obtained with plasmids containing 322 bp and 199 bp regions indicating that in this model the addition of the putative extended -10 motif of *pilE* has no impact on transcriptional activity of the upstream region; although it is possible that extended -10 promoter of *pilE* requires additional upstream sequence for efficient recognition by the σ^{70} RNAP holoenzyme, which was not present in this system. Analysis of extended -10 in *E. coli* and other species has demonstrated that promoters containing extended -10 contain often rely on additional transcription factors that bind upstream of the extended -10 for full activation. Since we only tested the activity of 322 bp upstream of *pilE*, it is possible that in *N. meningitidis* where the appropriate activators are present, the -10 motif could be functional.

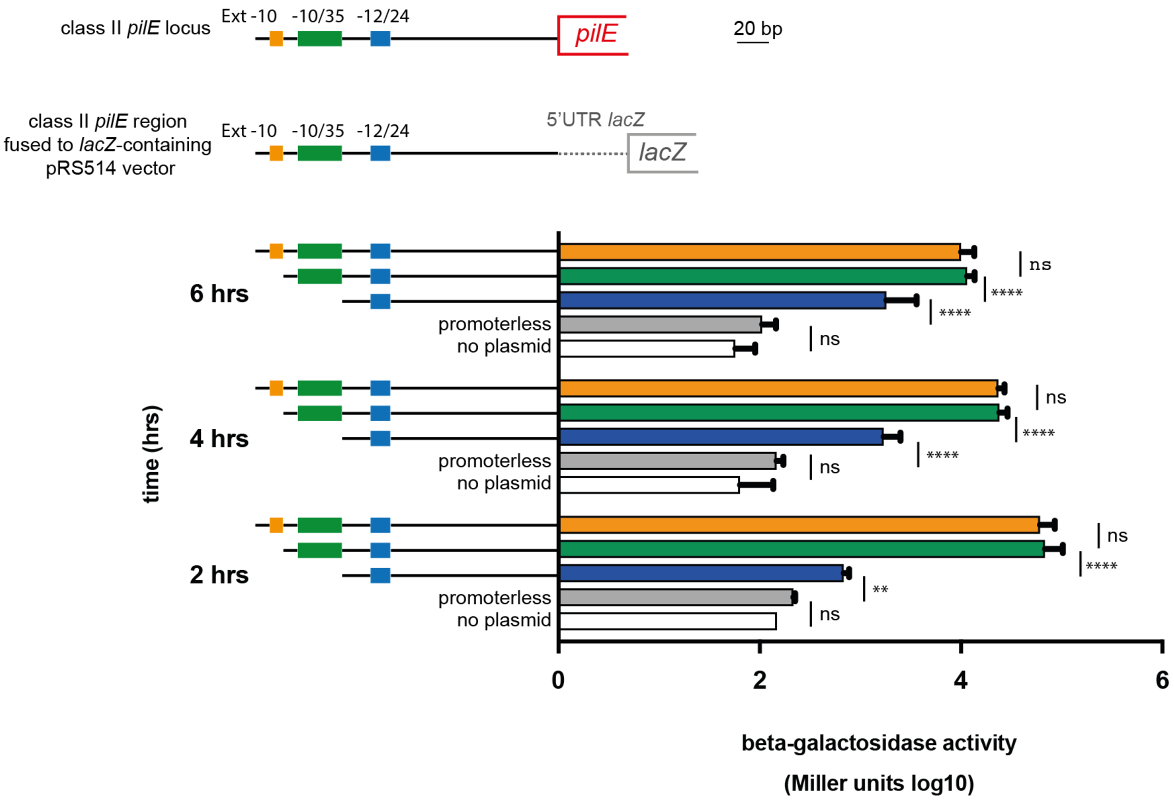


Figure 3.4 Class II *pilE* promoter activity in *E. coli*

Three promoter fragments were cloned into the pRS415 *lacZ* reporter plasmid and analysed for activity in *E. coli* DH5α. All three promoter sequences show β-galactosidase activity throughout growth. Two strains were used as controls: wild-type *E. coli* (no plasmid) and *E. coli* harbouring promoterless plasmid. The experiment was performed three times and analysed by two-way ANOVA where * $p \leq 0.05$, ** $p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ and ns is non-significant $p > 0.05$.

Interestingly, the 153 bp region possessing the -12/-24 element alone was active in *E. coli* although had reduced activity compared to the regions where the -10/-35 and extended -10 elements are present. σ^N-dependent transcription is known to require an activator that binds to the DNA upstream of the -12/-24 sequence and is brought to the σ^N-RNAP holoenzyme *via* DNA looping (as discussed in Section 1.5.1.4). Therefore, the reduced activity of the 155 bp *pilE* upstream region in *E. coli* could result from the lack of an activator in *E. coli* or/and the absence of the activator binding sequence in 155 bp sequence. Indeed, other reports have shown that the activity of pathogenic *Neisseria* class I *pilE* -12/-24 promoter is undetectable in *E. coli*, but can occur in the presence of *P. aeruginosa* pilin activator PilR. PilR binds to a specific upstream activator sequence (UAS) of the *P. aeruginosa* pilin gene, which is positioned 122 nucleotides away from transcriptional start site, to trigger σ^N-dependent pilin expression (Jin *et al.*, 1994). Based on homology to known PilR binding sites, putative UAS have been annotated 126 nucleotides upstream of *N. gonorrhoeae* class I *pilE* transcriptional start (Fyfe *et al.*, 1993). Furthermore, UAS has been shown to enhance *N. gonorrhoeae* class I *pilE* promoter activity when studied in *P. aeruginosa* (Carrick *et al.*, 1997), suggesting that PilR-dependent σ^N activation of *Neisseria pilE* -12/-24 promoter can occur. We therefore decided to examine whether UAS are present upstream of class II *pilE* and identify whether any possible transcription factors could affect pilin expression.

3.4 Investigating the presence of UAS and the role of candidate transcription factors in class II pilin expression.

UASs are present upstream of σ^N-dependent promoters and there can be multiple UASs, which bind cognate bEBPs (Studholme *et al.*, 2000). bEBPs recognise UASs *via* a HTH motif in their C-terminus, and usually bind as hexamers (Contreras *et al.*, 1988). Interestingly unlike *N. gonorrhoeae* and class I *pilE* expressing *N. meningitidis* which both have one predicted UAS upstream of *pilE* (Carrick *et al.*, 1997), commensal *N. elongata pilE* promoter contain three putative *pilE* UASs, which differentially affect commensal σ^N-

dependent promoter activity (Rendon *et al.*, 2013). Furthermore, *N. elongata* was reported to contain a homologue of *P. aeruginosa* PilR termed Neisseria pil activator (Npa) protein that recognises one of the UASs, and is required for pilin expression (Rendon *et al.*, 2013). We therefore decided to examine whether class II *pilE* contains an UAS and whether *N. meningitidis* contains homologue of Npa.

3.4.1 Class II *pilE* promoter contains a putative UAS in *N. meningitidis*

We used the reported PilR binding sequence of *P. aeruginosa* to conduct bioinformatic analysis of the upstream sequence of *pilE* in 77 class II *pilE* expressing meningococcal isolates. Three isolates were excluded due to the poor sequence of the contig harbouring this region. Based on the analysis of 74 isolates, we found that the position of an UAS relative to promoter elements is different in class II *pilE* expressing isolates compared to *N. gonorrhoeae* or class I containing *N. meningitidis* (Figure 3.5). Furthermore, the UASs are highly conserved among 74 isolates suggesting that this sequence might play a role in class II *pilE* regulation. Similar to *N. gonorrhoeae* MS11 and meningococcal 8013 class I *pilE* UAS, putative class II *pilE* UAS shares 60% sequence identity with the PilR binding site.

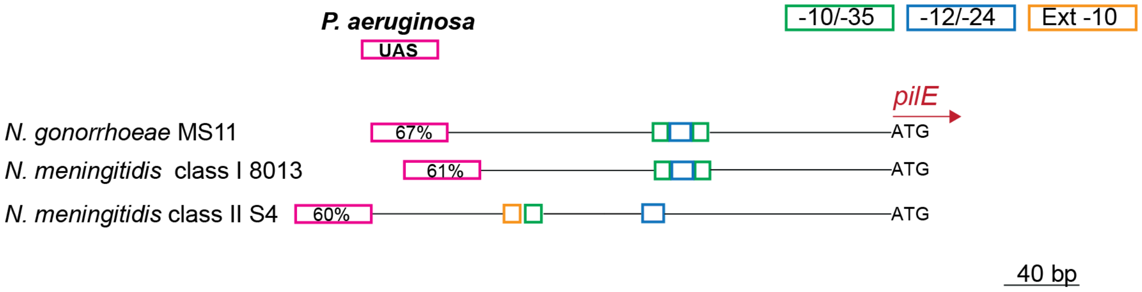


Figure 3.5 UAS comparison in *Neisseria*

Position of putative UAS relative to promoter elements in *N. gonorrhoeae*, class I *pilE* expressing *N. meningitidis* 8013 and 74 class II *pilE* expressing *N. meningitidis* isolates represented by strain S4. UAS was annotated based on the sequence identity (% values are shown in pink boxes) to a known *P. aeruginosa* UAS (Fyfe *et al.*, 1993).

Having identified putative UAS we next aimed to identify any putative bEBPs that could bind UAS and regulate *pilE*. bEBPs are transcription factors (TF) that bind to UAS of the -12/-24 promoters and are generally divided into five groups based on their structure (Wigneshweraraj *et al.*, 2005). bEBP contain three domain: 1) a central AAA+ domain (C) that is conserved among all bEBPs and is involved in the hydrolysis of ATP necessary for σ^N activation, 2) a C-terminal HTH-containing DNA binding domain (D) that is present in four out of five bEBP groups (Pelton *et al.*, 1999) and 3) a regulatory domain (R) present in some bEBP at the N-terminus which exhibits little or no homology between the bEBP groups and is responsible to bEBP activation in response to diverse range of signals (Figure 3.6) (Studholme & Buck, 2000). A characteristic sequence of the conserved C domain is GAFTGA motif that interacts directly with σ^N (Bordes *et al.*, 2003). Mutagenesis studies of bEBP showed that the GAFTGA domain is essential for ATP hydrolysis and σ^N-dependent transcription (Gonzalez *et al.*, 1998; Li *et al.*, 1999)

No GAFTGA-containing bEBPs have been reported in *N. meningitidis* or *N. gonorrhoeae* (Studholme & Buck, 2000). In commensal *N. elongata*, the bEBP Npa has been identified based on the homology to PilR from *K. kingae* and *P. aeruginosa*, which regulates pilin expression (Ishimoto *et al.*, 1992; Rendon *et al.*, 2013). Our comparison of *N. elongata* Npa sequence with PilR revealed that Npa contains two putative key domains: 1) GAFTGA motif which contains five of the six consensus residues and is identical to PilR sequence (GSFTGA), and 2) HTH motif which shares 62% sequence similarity with PilR (Figure 3.6). Deletion of Npa in *N. elongata* leads to marked downregulation of *pilE* transcript levels and direct binding of Npa to UAS of *N. elongata pilE* promoter has been demonstrated by DNA-binding assays (Rendon *et al.*, 2013). BLASTn analysis of *N. meningitidis* FAM18, S4 and 8013 genomes revealed a homologue of Npa that shared 60% amino acid sequence identity with *N. elongata* Npa. Despite high sequence identity, commensal Npa is a larger protein containing 575 amino acids whereas putative meningococcal Npa is only 155 amino acid long. Furthermore, the meningococcal homologue does not contain a GAFTGA motif. However, it contains HTH motif which is highly homologous to *N. elongata* Npa (87%) (Figure 3.6), suggesting that DNA binding could take place.

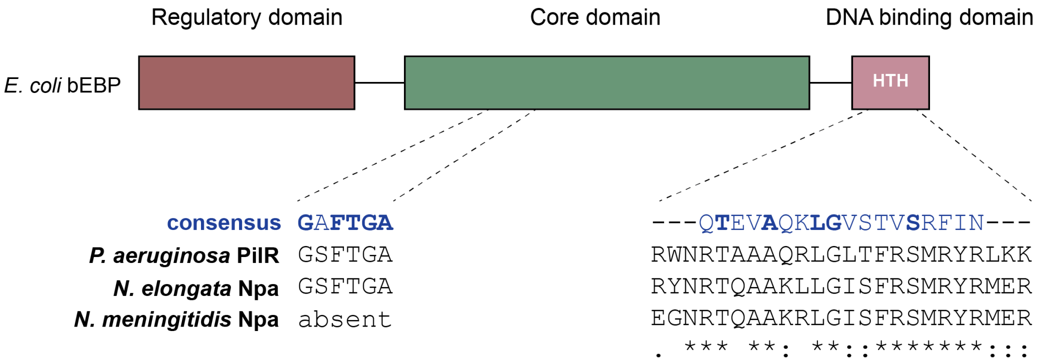


Figure 3.6 bEBP domain organisation and alignments of two domains of PilR and Npa

bEBPs typically contain three domains: regulatory domain that is an environmental sensor, conserved core AAA+ domain involved in ATP hydrolysis and transcription initiation, and DNA binding domain which contains a HTH. The core domain harbours the characteristic GAFTGA motif. *P. aeruginosa* PilR and *N. elongata* Npa contain a highly homologous GAFTGA domain (GSFTGA), which is absent in *N. meningitidis* Npa. Alignment of HTH domains shows high sequence homology between PilR, *N. elongata* and *N. meningitidis* Npa. The consensus GAFTGA and HTH sequences are shown in blue (Bordes *et al.*, 2003; Dodd *et al.*, 1990; Haydon *et al.*, 1991) and identical residues between consensus sequences and parts of the alignment are shown in bold blue.

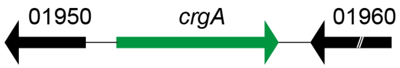
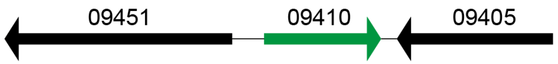
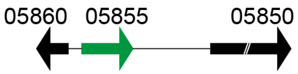
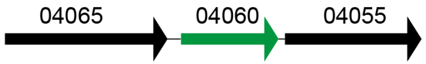
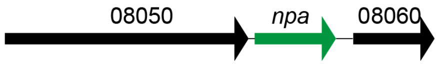
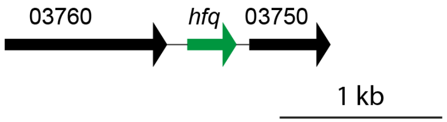
Since no GAFTGA-containing bEBP candidates have been reported in *N. meningitidis*, we decided to look for other TF that could potentially interact with the *pilE* promoter and affect transcription. There are >30 TFs found in *N. meningitidis* (Heidrich *et al.*, 2017). Among bacterial TF families, HTH-containing TFs form the largest and most well characterised group (Pabo *et al.*, 1992). We therefore decided to focus our analysis on TFs that contain a HTH motif. We reasoned that if a putative TF affects pilin expression, it is likely to impact bacterial adhesion to host cells as Tfp are the major adhesin of *N. meningitidis*. Therefore, out of the known TFs, we identified selected candidate factors that a) possess HTH motif and b) are known to be involved in regulating *N. meningitidis* adhesion to host cells. A total of six putative TFs that matched the parameters were selected based on a recent study by

Capel *et al.* (Capel *et al.*, 2016), which identified a number of genes involved in adhesion to epithelial and endothelial cells by transposon directed mutagenesis of class I *pilE*-expressing *N. meningitidis* Z5463. These included: methionine operon regulator *metR*, multiple transferrable resistance regulator *mtrR*, NMA2107, NMA1375, NMA1020 and contact-regulated gene A *crgA*. Tn insertion in *mtrR* leads to downregulation of meningococcal adhesion to epithelial cells whereas Tn insertion in either *metR*, NMA2107 or NMA1375 triggers upregulation of *N. meningitidis* adhesion to epithelial cells. Tn mutagenesis of NMA1020 resulted in downregulation of *N. meningitidis* binding to endothelial cells. In the same study *crgA* was identified as a negative regulator of meningococcal adhesion to epithelial cells which is in line with previous reports (Deghmane *et al.*, 2002). In addition to the six candidate TFs, we extended our analysis to investigate two additional regulators, Npa and Hfq. *N. meningitidis* homolog of *N. elongata pilE* regulator Npa described earlier (Figure 3.6) contains a putative HTH motif but has not been implicated in adhesion (Capel *et al.*, 2016). Hfq in turn does not contain HTH motif. It is an RNA binding protein and functions as a global post-transcriptional regulator of proteins involved in stress-response and metabolism in *N. meningitidis* (Pannekoek *et al.*, 2009). Hfq has been reported to modulate pilin expression in a number of class I *pilE* containing *N. meningitidis* isolates and interestingly the effect exerted by Hfq on class I pilin expression is strain-dependent. Deletion of *hfq* in class I *pilE* containing *N. meningitidis* H44/76 results in downregulation of pilin. The opposite effect is observed in a different class I *pilE* expressing *N. meningitidis* MC58: based on the proteomic analysis of WT and Δ*hfq* strains, pilin was shown to be one of the 20 proteins that were upregulated in the absence of *hfq* (Fantappiè *et al.*, 2009). Recently Hfq has been shown to regulate a small RNA NMnc0001 in class I *pilE* expressing *N. meningitidis* 8013. The expression of this sRNA correlated with class I *pilE* expression and therefore it was proposed to be involved in pilin regulation

(Heidrich *et al.*, 2017). The exact mechanisms of Hfq-dependent regulation of pilin remains unknown and its role in regulation of class II *pilE* has not been addressed before. Therefore we decided to include it in our analysis of possible *pilE* regulators.

We first examined whether the eight candidate genes (six candidate TFs, Npa and Hfq), which were originally identified in class I *pilE*-expressing *N. meningitidis* strains (8013 and Z5463) were also present in class II *pilE*-expressing *N. meningitidis* FAM18 and S4 (unpublished). Amino acid sequence identity was analysed and the candidate gene locus was determined using the annotated FAM18 genome (Table 3.1). Based on our analysis, all eight candidate genes were present in FAM18 and were near identical between two class II *pilE*-expressing *N. meningitidis*. Furthermore, they exhibited high sequence homology with their counterparts found in class I *pilE*-expressing isolates (Table 3.1), indicating that these genes are conserved across *N. meningitidis*.

Table 3.1 Candidate TFs in class II *pilE* expressing *N. meningitidis*

Gene ID (FAM18)	Gene name*	Gene description*	Proposed function*	Amino acid sequence identity** FAM18 *vs.* S4	FAM18 *vs.* 8013	Z5463 *vs.* 8013	Genomic context in FAM18 and S4
NMC_RS01955	*crgA*	HTH-type transcriptional regulator	Tn disruption decreases adhesion to epithelial cells	100%	100%	99%	
NMC_RS10785	*metR*	HTH-type transcriptional regulator	Tn disruption decreases adhesion to epithelial cells	100%	96%	99%	
NMC_RS08680	*mtrR*	HTH-type transcriptional regulator	Tn disruption increases adhesion to epithelial cells	100%	100%	99%	
NMC_RS09410		HTH-type transcriptional regulator	Tn disruption increases adhesion to epithelial cells	100%	100%	100%	
NMC_RS05855		HTH-type transcriptional regulator	Tn disruption increases adhesion to epithelial cells	99%	99%	98%	
NMC_RS04060		HTH-type transcriptional regulator	Tn disruption increases adhesion to endothelial cells	100%	98%	100%	
NMC_RS08055	*npa*	Two-component system transcriptional regulator	activates *pilE* expression in *N. elongata*	100%	99%	100%	
NMC_RS03755	*hfq*	global post-transcriptional regulator	regulates pilin expression in strain-dependent manner	98%	99%	100%	

8013 and Z5463: class I *pilE* expressing *N. meningitidis*

FAM18 and S4: class II *pilE* expressing *N. meningitidis*

*Gene name, description and proposed function were assigned based on the annotation of homologues found in 8013 (Vallenet *et al.*, 2006) and Z5463 (Capel *et al.*, 2016)

**percentage identitity determined by CLUSTAL alignment of amino acid seqeunces retreived from pubMLST database

3.4.2 Analysis of pilin levels in Δ*hfq* and transcription factor knockout backgrounds

Having identified the presence of the conserved candidate genes in S4, we then aimed to test whether these putative factors are involved in modulating pilin expression.

To construct mutant strains in class II *pilE* containing *N. meningitidis* S4, two approaches were used: 1) insert a transposon into the coding sequence of the candidate gene, or 2) perform a complete deletion of the gene by replacement of the open reading frame (ORF) with a kanamycin resistance cassette. S4 homologues of *mtrR*, *metR*, NMC_RS09410, NMC_RS08680 were previously inactivated in *N. meningitidis* 8013 by Tn insertion (Geoffroy *et al.*, 2003). The DNA regions flanking the transposon were amplified by PCR from the genomic DNA from 8013 containing Tn mutants, and the resulting PCR fragments were used for transformation into S4. The length of the homologous regions was determined by sequence comparison between 8013 and S4 loci and only conserved sequences were amplified to minimise the variation within the S4 locus upon transformation into S4. The resulting strains harbouring transposon insertions included S4Δ*mtrR*, S4Δ*metR*, S4Δ*0914* and S4Δ*04060*. The ORFs of *npa*, *hfq*, *crgA* and NMC_RS04060 were replaced with a kanamycin resistance cassette to yield S4Δ*npa*, Δ*hfq*, Δ*crgA* and Δ04060.

Mutant strains and wild-type (WT) S4 were grown overnight on solid medium and cell lysates were collected to examine pilin expression by Western blotting with anti-pilin antibody. No apparent differences in pilin protein levels could be detected in any of the mutants apart from Δ*hfq* compared to the WT pilin levels (Figure 3.7). However, this conclusion is based on single biological repeat and quantification of the Western blotting was not performed, as anti-RecA antibody used to detect the expression of the RecA loading control resulted in high background activity. Thus, experiments are under way to analyse pilin expression in these backgrounds with optimised loading control and additional biological samples.

Based on one biological repeat we observe that the deletion of *hfq* lead to reduction in level of pilin detected by Western blotting. This is in line with the observations in class I *pilE* containing *N. meningitidis* H44/76 (Pannekoek *et al.*, 2009). It remains to be determined whether such downregulation is reproducible and whether this effect can be observed in other class II *pilE* expressing isolates, considering that strain-dependent effect of Hfq on pilin has previously been observed in class I *pilE* expressing meningococcus. No changes in pilin levels were recorded in S4Δ*npa* (n=1) suggesting that in *N. meningitidis* Npa has a distinct function compared to *N. elongata* Npa which functions as *pilE* activator.

Overall our analysis of candidate regulators of class II pilin is not comprehensive as we studied only a single condition. Furthermore, the candidate transcription factors may be involved in regulation of pilin at the transcription level as we did not examine the *pilE* transcript levels in these strains.

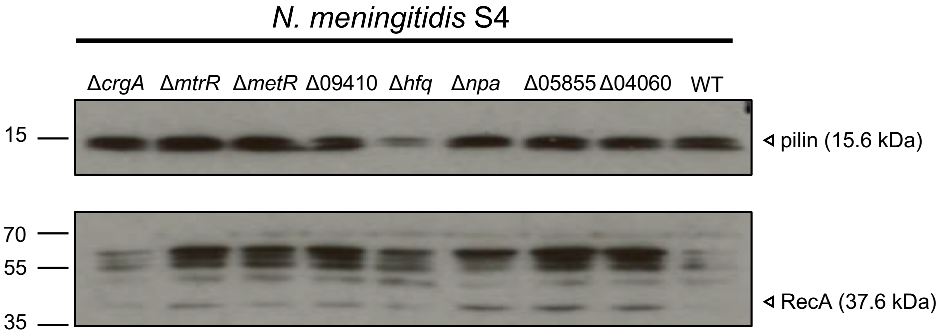


Figure 3.7 Expression of pilin in class II *pilE-expressing N. meningitidis*

Western blot analysis of pilin levels in S4 wild-type (WT), S4Δ*crgA*, S4Δ*mtrR*, S4Δ*metR*, S4Δ09410, S4Δ*hfq*, S4Δ*npa*, S4Δ05855 and S4Δ04060. Cell lysates were collected after overnight growth of N. *meningitidis* on BHI plate and separated on 12% polyacrylamide gel. Pilin expression was detected using anti-pilin antibodies. RecA detection was used as a loading control however the antibody used resulted in cross-reaction therefore quantification of Western blotting based on the loading control could not be performed. The experiment was performed once.

Summary

In this chapter we focused on the analysis of class II *pilE* promoter elements. We only considered promoter elements that match the canonical σ-dependent promoter sequences. However, a promoter is a combination of different structural features (Browning & Busby, 2016) and therefore it is possible that there are other elements within class II *pilE* upstream region that may serve as regulatory elements in addition to the annotated consensus promoters.

Bioinformatic analyses revealed conserved putative σ^{70} and σ^{N}-dependent promoter elements in 77 class II *pilE*-expressing *N. meningitidis* isolates. The presence and position of the class II *pilE* -12/-24 sequence was interesting as unlike in the promoter region of class

I *pilE*, -12/-24 sequence does not overlap with the -10/-35 sequence in the class II *pilE* promoter region. Furthermore, our analysis of class II *pilE* upstream sequences in *E. coli* demonstrated that the class II *pilE* upstream region containing just putative -12/-24 element is sufficient to trigger *lacZ* expression, suggesting that this sequence is functional in transcription activation. Analysis of the genomes from 290 meningococcal isolates showed that they harbour a gene encoding σ^N lacking HTH but containing RpoN box. This led us to speculate that recognition of -12/-24 element of the -12/-24 sequence in the promoter of class II *pilE* by σ^N could be possible.

The upstream sequence of class II *pilE* retains an UAS that is homologous to *P. aeruginosa* pilin UAS, which is known to bind the pilin regulator PilR in this species. The presence of UASs suggests that bEBP may be required for σ^N-dependent transcription of class II *pilE*. No GAFTGTA-containing bEBPs have been reported in of *N. meningitidis* 8013 and FAM18, making it challenging to study the role of putative bEBPs in class II *pilE* regulation. The absence of the canonical bEBP in meningococcal genomes could imply that either a) σ^N-dependent transcription is not active in *Neisseria*, or b) meningococcal σ^N relies on alternative σ^N activators, or c) σ^N in *N. meningitidis* functions in the absence of bEBP in a non-canonical manner known as σ^N bypass transcription. Indeed in *E. coli* σ^N bypass transcription occurs by recognition of the -12/-24 promoters including those with a non-canonical -12 element (Schaefer *et al.*, 2015). Considering that meningococcal σ^N lacks a HTH motif that mediates the recognition of -12 sequence, the bypass form of σ^N could be employed by meningococcal σ^N.

We considered putative TFs candidates that could be involved in pilin regulation. We constructed *N. meningitidis* S4 mutants lacking the homologs of the *N. elongata* Npa, Hfq or other putative TFs that we identified based on two criteria: a) they contain a HTH DNA binding domain and b) they have previously been implicated in modulating adhesion in *N. meningitidis* (Capel *et al.*, 2016). Apart from Hfq deletion that lead to reduction in pilin protein levels, mutation of the candidate genes had no effect on pilin expression based on Western blotting analysis of pilin levels. However the loading control could not be used to perform the accurate quantification and the experiment was performed once. We cannot exclude the possibility that these putative factors could still be implicated in pilin regulation under conditions that were not examined here. Furthermore, it is possible that other TFs, which were not studied here, could regulate *pilE* transcription. One approach to identify putative TF that regulate *pilE* at the class II *pilE* locus would be to employ a recently reported method of genomic locus proteomics (GLoPro) (Myers *et al.*, 2018). The method utilises a single guide RNA corresponding to the locus of interest and dCas9 fused with protein labelling compound. Upon activation and subsequent proximal protein labelling, proteomic analysis is performed to identify the number and types of protein associated with a specific locus. This approach, when applied to class II *pilE* locus analysis, could reveal the TFs associated with *pilE* and help validate whether putative UAS identified upstream of class II *pilE* is involved in interaction with any regulators. It would also allow to examine whether there are any additional activator/repressor binding sites within class II *pilE* upstream region.

Identifying conserved promoter elements in class II *pilE*-expressing isolates, confirming the functional activity of the -12/-24 and -10/-35 sequences of *pilE* and identification of a meningococcal σ^N that possess RpoN box and therefore could still retain binding, raises an important question about which promoters upstream of class II *pilE* are functional in *N. meningitidis*.

4 Results: RpoN is not involved in class II *pilE* regulation in *N. meningitidis*

In the previous chapter bioinformatic analysis demonstrated that the class II *pilE* promoter sequence, including the -12/-24 sequence, are conserved among 76 *N. meningitidis* isolates. In *N. gonorrhoeae* and class I *pilE*-expressing *N. meningitidis* it has been shown that the -12/-24 promoter is not involved in pilin transcriptional activity (Carrick *et al.*, 1997; Fyfe *et al.*, 1995). However, several lines of evidence suggest that σ^N-dependent promoter could be active in class II *pilE* containing *N. meningitidis*: 1) meningococcal genome analysis revealed that the majority of *N. meningitidis* isolates with class I or class II *pilE* harbour an *rpoN* gene that encodes a σ^N lacking HTH but containing the -24 biding RpoN box, which suggests that promoter binding could occur, 2) the -12/-24 sequence does not overlap with -10/-35 promoter sequence in isolates with class II *pilE* and 3) class II *pilE* promoter fusions demonstrated that the promoter fragment containing only the -12/-24 element is functional in *E. coli*. We therefore examined whether meningococcal σ^N is functional and if it can contribute to class II *pilE* expression.

4.1 *rpoN* transcript but not the σ^N protein can be detected in *N. meningitidis*

First we investigated whether σ^N is expressed in *N. meningitidis*. Initially we determined *rpoN* transcript levels in the class II *pilE* containing *N. meningitidis* strain S4. As a control, S4Δ*rpoN* strain was constructed which carried a kanamycin resistance cassette in place of the *rpoN* ORF. Wild-type (WT) S4 and S4Δ*rpoN* were grown in liquid BHI for 24 hours, and growth was monitored by measuring the OD_{600} of the cultures. Growth of the two strains was similar (Figure 4.1A), indicating that the absence of *rpoN* does not impact bacterial fitness. Specific time points were chosen to collect total RNA, representing particular growth phases: early-, mid-, late- logarithmic and stationary phases were represented by 3, 6, 8 and 22 hours respectively. qRT-PCR was used to measure relative expression of *rpoN*. Analysis demonstrated that *rpoN* is expressed and is present throughout bacterial growth although at low levels relative to the standard control tmRNA (Figure 4.1B). *rpoN* transcript varied according to the growth phases with the highest expression in stationary phase (22 hours) (Figure 4.1B). No transcript was detected in strains lacking *rpoN*.

Next we determined whether σ^{N} is detected in S4. S4$\sigma^{N\text{-His}}$ was constructed which harboured a gene encoding a C-terminal His-tagged σ^{N}, in place of the wild-type *rpoN*. Bacteria were grown overnight on solid media and cell lysates were collected and analysed by Western blotting. No protein corresponding to the expected size of $\sigma^{N\text{-His}}$ (31.5 kDa) was detected in S4$\sigma^{N\text{-His}}$ (data not shown) implying that either *rpoN* mRNA is not translated under the conditions tested or that the amount of σ^{N} is below the limit of the detection.

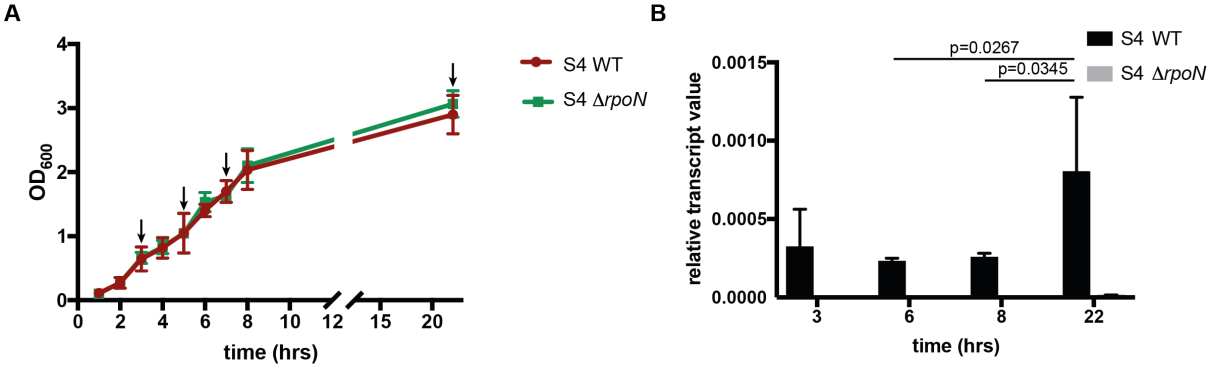


Figure 4.1 Presence of *rpoN* transcript in class II *pilE N. meningitidis*

A. *N. meningitidis* S4 WT and Δ*rpoN* were grown at 37°C in liquid BHI medium. Growth was monitored at OD$_{600}$. Arrows on the curve represent the time points when RNA was collected for transcript analysis. Error bars indicate SD. B. *rpoN* expression was measured by qRT-PCR at different time points (3, 6, 8 and 22 hrs). The amount of transcript is represented as the R value standardised to tmRNA transcript levels. Two-way ANOVA was used to perform comparison of relative transcript levels in WT S4. There was a significant difference between *rpoN* transcript levels in WT S4 at 22 hrs and 8 hrs (p=0.0345); and 22 hrs and 6 hrs, (p=0.0267). No transcript was detected in S4Δ*rpoN*. The experiment was performed three times using independent biological replicates, analysed in triplicate.

4.2 *N. meningitidis* σ^N does not function as a canonical σ^N-dependent promoter activator when tested in *E. coli*

Given that σ^N was not detected in *N. meningitidis*, we utilized *E. coli* to determine the activity of *N. meningitidis* σ^N. Figure 4.2A shows the two vector system that was designed for this analysis: one vector contains *rpoN* under the control of an arabinose inducible promoter, while the second vector harbours a known *E. coli* σ^N-dependent promoter for the phage shock protein A (*pspA*) gene located upstream of *lacZ* reporter. *pspA* is part of the *pspA*BCDE operon and is a well characterised member of *E. coli* σ^N-dependent regulon (Brissette *et al.*, 1991). P*pspA* contains the σ^N-dependent promoter elements: 1) a canonical

-12/-24 sequence that binds σ^N; 2) upstream activator sequence (UAS) that binds the *E. coli* bEBP PspF; and 3) integration host factor (IHF) binding site recognized by IHF, which brings together bEBP and σ^N-RNAP (Figure 4.2B) (Jovanovic *et al.*, 1997). In the system described in Figure 4.2A *rpoN* expression is induced in the presence of arabinose and its ability to activate transcription is assessed using the activity of P*pspA*-*lacZ* as a reporter. To construct the system, *E. coli* MG1655 lacking its endogenous copy of *rpoN* was generated (MG1655Δ*rpoN*). The growth of this strain was assessed in liquid media (Figure 4.2C). The absence of *rpoN* in *E. coli* renders it auxotrophic for glutamine as *glnA*, which encodes glutamine synthetase, is controlled by σ^N (Reitzer *et al.*, 1987). Therefore, growth of all *E. coli* MG1655Δ*rpoN* strains was supplemented with 0.2% glutamine in subsequent experiments to ensure that the growth of the mutant is comparable to WT MG1655. Next, markerless deletion of *lacZ* was performed in MG1655Δ*rpoN* to remove the endogenous β-galactosidase activity for the subsequent analysis of σ^N activity. MG1655Δ*rpoN*Δ*lacZ* was then transformed with pBAD33 carrying *N. meningitidis rpoN* (pBAD33-$rpoN^{Nm}$), and pRS415 harbouring *lacZ* as a reporter under the control of P*pspA* (pRS415-P*pspA*). MG1655Δ*rpoN*Δ*lacZ* containing pBAD33 expressing *E. coli rpoN* (pBAD33-$rpoN^{E.coli}$) and pRS415-P*pspA*, served as a positive control. Negative controls to detect background reporter activity, included four MG1655Δ*rpoN*Δ*lacZ* derivatives: strain 1 and 2 harboured promoterless pRS415 in combination with either pBAD33-$rpoN^{Nm}$ or pBAD33-$rpoN^{E.\ coli}$ respectively, strain 3 contained pRS415-P*pspA* with empty pBAD33 (without *rpoN*) and strain 4 contained empty pBAD33 and promoterless pRS415.

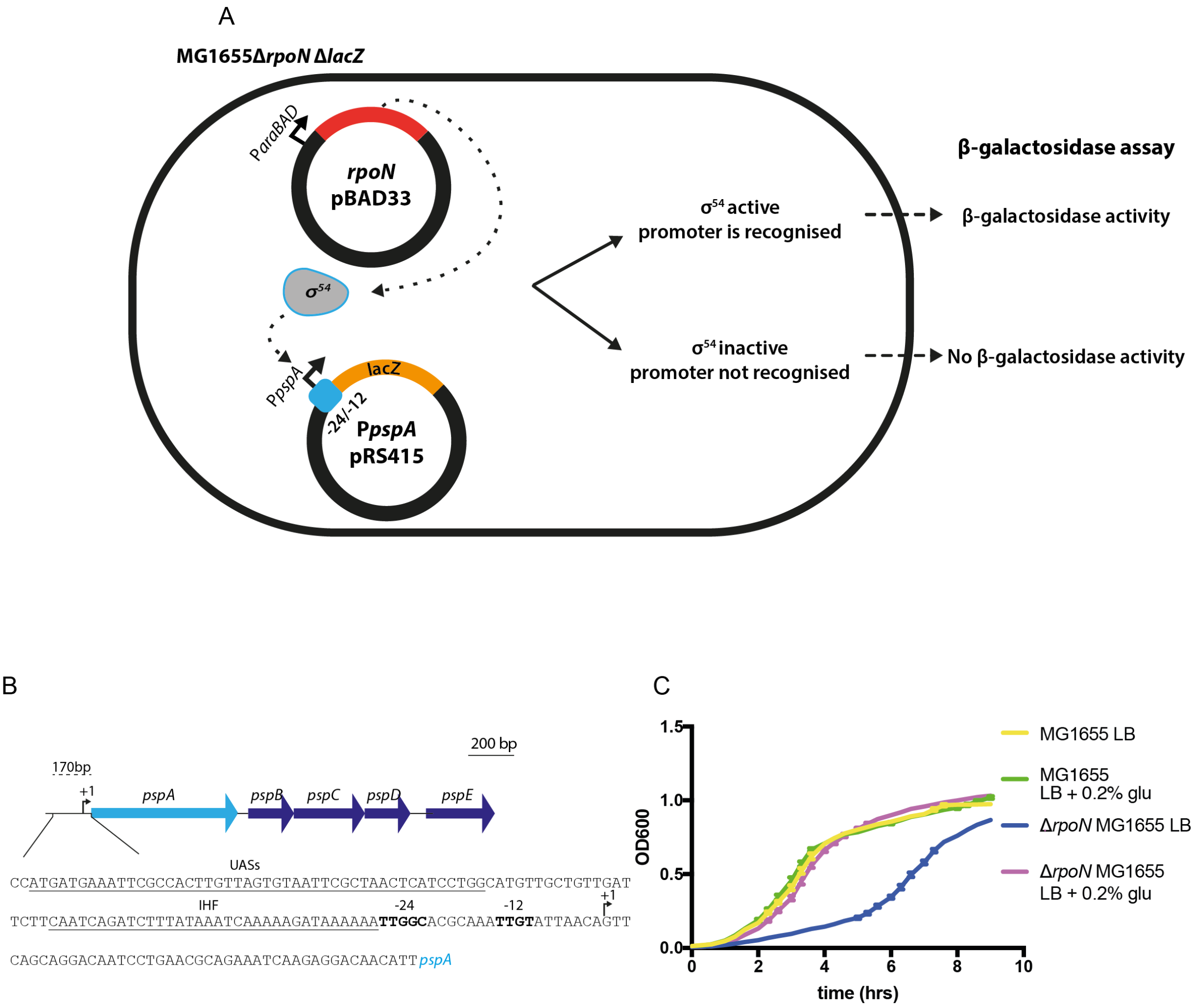


Figure 4.2 *E. coli* two vector system for functional analysis of σ^N

A. Schematic diagram showing reporter plasmid (pRS415-P*pspA*) and *rpoN* expression plasmid (pBAD33-*rpoN*) in MG1655Δ*rpoN*Δ*lacZ* designed for analysis of σ^N activity using β-galactosidase assay. B. Organisation of MG1655 *pspABCDE* operon and *pspA* promoter region (adapted from Model 1997). 170 bp of the upstream region of *pspA* is shown. -12/-24 promoter is highlighted in black, UAS and IHF binding site are underlined. C. Growth curve of MG1655 WT and MG1655ΔrpoN grown at 37°C in LB with or without 0.2% glutamine (glut).

Prior to performing β-galactosidase assays in *E. coli*, the system was validated by detecting σ^N expression from pBAD33 in response to arabinose induction. MG1655Δ*rpoN*Δ*lacZ* containing pRS415-P*pspA* and pBAD33-*rpoN*$^{E. coil}$ or pBAD33-*rpoN*^{Nm} both carrying *rpoN* with a hemagglutinin (HA) tag at the C-terminus were grown at 37°C overnight. Overnight cultures were used to inoculate fresh media containing 0.02% arabinose to induce *rpoN* expression and growth was monitored over the next 4 hrs (Figure 4.3A). Cell lysates were prepared at time points 1.5, 2.5 and 4 hrs after induction. σ^N expression was assessed by Western blotting using anti-HA antibodies (Figure 4.3B). Interestingly meningococcal σ^N expression from pBAD33 was only detectable at 1.5 and 2.5 hrs after induction, whereas *E. coli* σ^N was present at all time points in the presence of arabinose. For subsequent experiments, samples were collected at mid-exponential phase (2.5 hrs post induction) as the highest amounts of *E. coli* and *N. meningitidis* σ^N was detected at this time point (Figure 4.3B). Using the β-galactosidase assay, we observed the activity of *pspA* promoter with *E. coli* σ^N. Upon induction with arabinose, *E. coli* σ^N led to a 1.5 fold upregulation of P*pspA* compared to the isogenic strain where no arabinose was added (p=0.007, Figure 4.3C). Of note, the activity of P*pspA* with *E. coli* σ^N in the absence of arabinose is significantly higher than the control strains (empty pBAD33 vector and pRS415-P*pspA*), which suggests that the uninduced expression of pBAD33-*rpoN*$^{E.coli}$ observed in Figure 4B is sufficient to activate P*pspA* (Figure 4.3C). There was no detectable σ^N-dependent β-galactosidase activity when *N. meningitidis* σ^N was induced, as β-galactosidase levels in this strain were equal to background activity in the control strain harbouring empty pBAD33 and pRS415-P*pspA* (Figure 4.3C). This suggests that in this system, meningococcal σ^N is non-functional as an activator of transcription at the -12/-24 sequence.

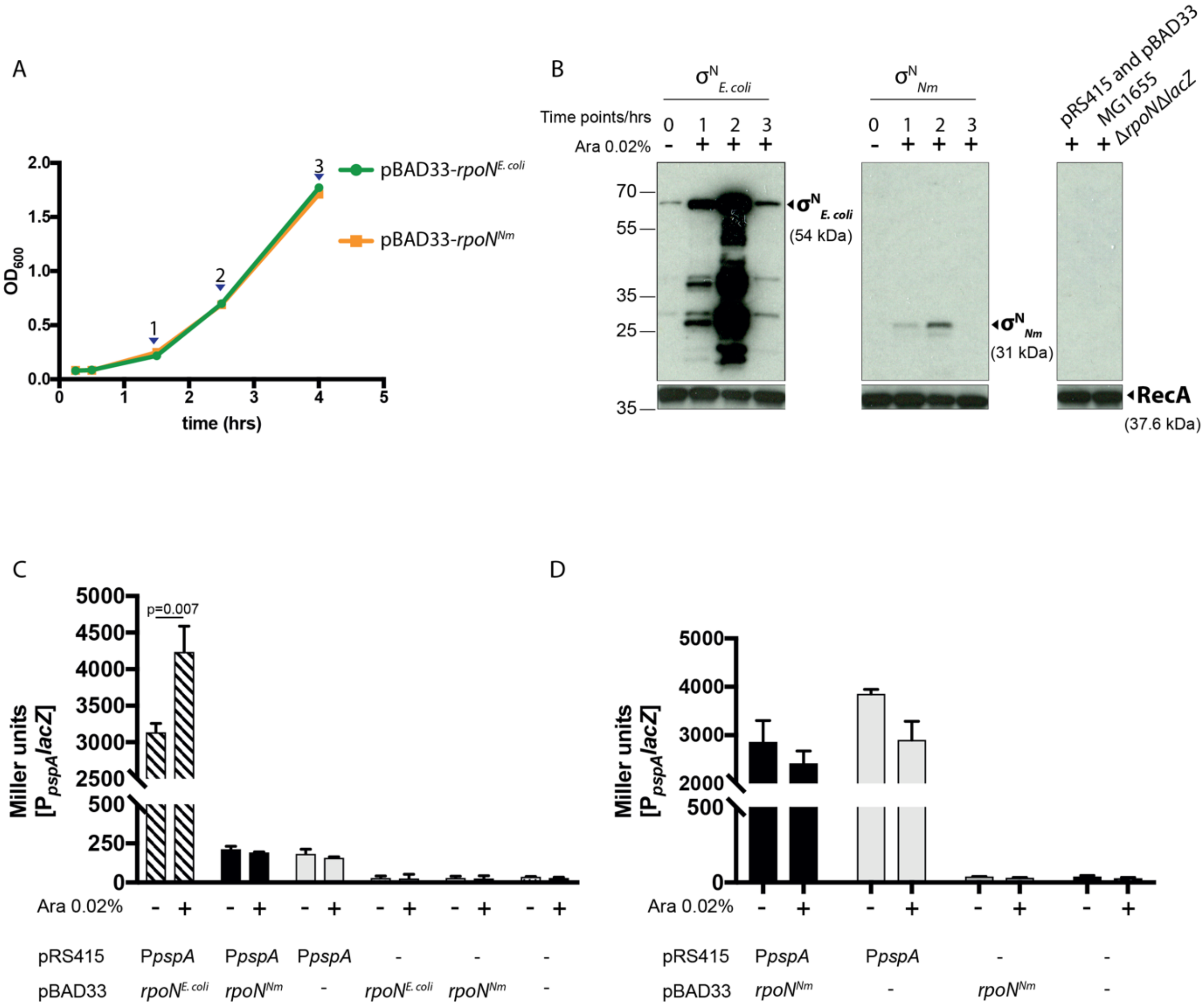

A
2.0
1.5
1.0
0.5
0.0
OD600
0
1
2
3
4
5
time (hrs)
1
2
3
pBAD33-rpoNE. coli
pBAD33-rpoNNm
B
σN E. coli
σN Nm
pRS415 and pBAD33
MG1655
ΔrpoNΔlacZ
Time points/hrs
0 1 2 3
0 1 2 3
Ara 0.02%
- + + +
- + + +
+ +
70
55
35
25
35
σN E. coli
(54 kDa)
σN Nm
(31 kDa)
RecA
(37.6 kDa)
C
5000
4500
4000
3500
3000
2500
500
250
0
p=0.007
Miller units
[PpspA/lacZ]
Ara 0.02%
- + - + - + - + - + - +
pRS415
PpspA
PpspA
PpspA
-
-
-
pBAD33
rpoNE. coli
rpoNNm
-
rpoNE. coli
rpoNNm
-
D
5000
4000
3000
2000
500
0
Miller units
[PpspA/lacZ]
Ara 0.02%
- + - + - + - +
pRS415
PpspA
PpspA
-
-
pBAD33
rpoNNm
-
rpoNNm
-

Figure 4.3 Functional analysis of *N. meningitidis* σ^N in *E. coli*

A. Growth of MG1655Δ*rpoN*Δ*lacZ* expressing either *E. coli* σ^N or *N. meningitidis* σ^N from pBAD33 at 37°C in LB supplemented with 0.02% arabinose and 2% glutamine. Triangles indicate the time points in hours at which the samples were collected for the analysis of σ^N expression (time point 1, 2, 3 represent 1.5, 2.5 and 4 hrs respectively). B. Western blot analysis of σ^N in MG1655Δ*rpoN*Δ*lacZ* expressing either *E. coli* σ^N (σ$^N_{E.\ coli}$) or *N. meningitidis* σ^N (σ$^N_{Nm}$) from pBAD33 system described in A. before arabinose induction (time point 0) and after the induction with arabinose at time points 1, 2 and 3 as described in A. σ factor expression is detected with anti-HA antibodies and RecA is used as a loading control. Blot is a representative of three biological repeats. C. β-galactosidase activity from P*pspA* with either *E. coli* or *N. meningitidis* σ^N, or no σ^N in MG1655Δ*rpoN*ΔlacZ. Activity is expressed in Miller units. P*pspA* activity is increased in the presence of *E. coli* σ^N (p=0.007, Student's t-test). Experiments were performed three times with three technical replicates on each occasion. D. σ^N-dependent P*pspA* promoter activity in the presence of *N. meningitidis* and endogenous *E. coli* σ^N in MG1655Δ*lacZ*. P*pspA* activity remains unchanged in the presence of induced *N. meningitidis* σ^N. Experiment is a representative of two biological repeats. Error bars indicate SD.

There are three potential explanations for this result. Firstly, the absence of P*pspA* activity with meningococcal σ^N could be due to the inability of *N. meningitidis* σ^N to form functional interactions with *E. coli* RNAP. Secondly, *N. meningitidis* σ^N lacks HTH motif thus P*pspA* recognition and binding might be impaired. Thirdly, providing meningococcal σ^N binds to the promoter, transcription may not take place because *N. meningitidis* σ^N may require a cognate *Neisseria*-specific σ^N activator. If this is correct, then *N. meningitidis* σ^N could repress transcription once bound to *E. coli* -12/-24 promoter. To address this possibility, we measured the β-galactosidase activity in the presence of both *E. coli* σ^N and *N. meningitidis* σ^N. If meningococcal σ^N is indeed a repressor, then once bound, it would occupy the promoter thereby preventing *E. coli* σ^N-dependent activation of P*pspA*. We utilized MG1655Δ*lacZ* containing pRS415-P*pspA* which harbours an intact endogenous copy of *E.*

coli rpoN on the chromosome and *N. meningitidis* σ^N on pBAD33 vector. We observed the activity of P*pspA* in the presence of empty pBAD33 suggesting that endogenous *E. coli* σ^N levels are sufficient to activate P*pspA* (Figure 4.3D). Interestingly, there was no change in the expression of P*pspA-lacZ* when *N. meningitidis* σ^N was induced, indicating that meningococcal σ^N does not repress *E. coli* σ^N-dependent activation of P*pspA* (Figure 4.3D). Therefore *N. meningitidis* σ^N does not function as an activator or a repressor of canonical σ^N-dependent *pspA* promoter in *E. coli.*

4.3 Pilin does not vary in the presence or absence of σ^N in *N. meningitidis*

Analysis of the activity of meningococcal σ^N in *E. coli* suggests that meningococcal σ^N is unable to interact with the canonical -12/-24 σ^N binding sequence. However, to exclude that this was due to the absence of putative meningococcal σ^N activators or optimal promoter arrangement, we next analysed whether meningococcal σ^N regulates class II *pilE*.

First we examined the activity of putative class II *pilE* promoters using *lacZ* fusions. The *lacZ* gene was used to replace the ORF of *pilE* leaving the *pilE* promoter sequence including 5' UTR intact (P*pilE-lacZ*). We introduced this P*pilE-lacZ* transcriptional fusion at the native *pilE* locus in S4 and S4Δ*rpoN*. The resulting strains S4Δ*pilE*::P*pilE-lacZ* and S4Δ*rpoN*Δ*pilE*::P*pilE-lacZ* were plated onto solid BHI media containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), and the detection of blue colonies was used as a read out to determine the activity of the promoter. After overnight growth, visual inspection of bacterial plates revealed that all strains including S4Δ*rpoN*Δ*pilE*::P*pilE-lacZ* appeared blue on the plates suggesting that *rpoN* is not required for *pilE* expression (Figure 4.4A).

Consistent with this observation, *rpoN* deletion had no effect on *pilE* transcript levels throughout bacterial growth as measured by qRT-PCR (Figure 4.4B). Interestingly, the relative expression of class II *pilE* was independent of the growth phase under these conditions. Collectively these results demonstrated that in *N. meningitidis* σ^N is not required for pilin expression under the conditions tested.

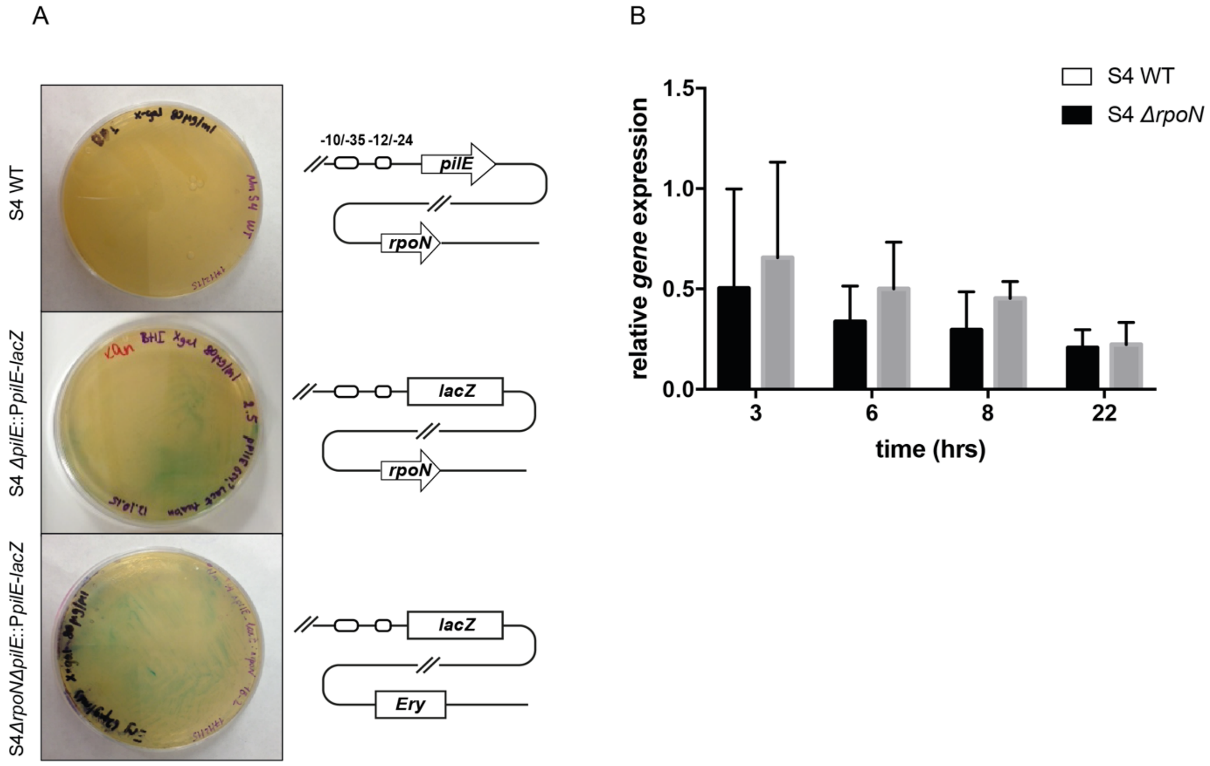


Figure 4.4 Effect of *rpoN* deletion on pilin levels in *N. meningitidis*

A. S4, S4Δ*pilE*::P*pilE-lacZ* and S4Δ*rpoN*Δ*pilE*::P*pilE-lacZ* strains were grown on a medium containing X-gal. Blue colonies indicate expression of ß-galactosidase. B. Strand-specific qRT-PCR analysis of pilin transcript at different time points during the growth in WT S4 and S4Δ*rpoN*. *pilE* mRNA is present throughout bacterial growth. The amount of transcript is presented as R value and normalized to tmRNA (n=3, error bars show SD calculated using two way ANOVA).

4.4 Identification of *pilE* transcriptional start site

If all promoters upstream of class II *pilE* are active, it is possible that the activation of one promoter element masks the transcription from the other. Thus we next mapped the class II *pilE* transcriptional start site (TSS) to determine which of the putative promoter elements are involved in transcription initiation of the class II *pilE* gene. Total RNA was prepared from *N. meningitidis* S4 grown in liquid BHI at 37°C to mid-exponential phase. Figure 4.5A shows the region upstream of class II *pilE* containing putative promoter elements, and the primers (P1 and P2) which were designed to capture potential transcripts generated from either or both the -10/-35 and -12/-24 promoters (P2), or only from the -10/-35 promoter (P1). Initially different amounts of total RNA were used to determine the optimal amount of template RNA needed to identify the TSS. A total of 1 µg and 2.5 µg of RNA was used to generate cDNA with radiolabelled P1 and P2; the products were analysed on a denaturing polyacrylamide gel. A control reaction was performed where radiolabelled tmRNA specific primer was used for cDNA synthesis and the product was analysed on the gel. The control reaction resulted in a ~40 nt transcriptional product (C) corresponding to the TSS of the tmRNA as previously reported (Heidrich *et al.*, 2017). However additional smaller products were detected, possibly due to cross hybridization of the probe with other transcripts (Figure 4.6B). The σ^{70}-dependent -10/-35-associated product (P1a, ~35 nt) was obtained with primer P1 (Figure 4.5B). Similarly, a ~74 nt product was generated with the P2 primer (P2b), also corresponding to a TSS associated with the putative -10/-35 promoter. Interestingly, a ~68 nt product (P2b') was generated with the P2 primer. However, at present it is not clear whether this is an alternative TSS associated with the -10/-35 promoter (mapped 14 nt away from -10 box) or is a degradation product (Figure 4.5B).

Importantly, no product of ~38 nt was detected using primer P2 indicating that the class II *pilE* region containing -12/-24 promoter is not involved in class II *pilE* transcriptional activation under these conditions.

To determine the nucleotide sequence of the extension products a dideoxy chain termination sequencing reaction with P2 was run in parallel using a PCR fragment of the upstream region of class II *pilE* as the template. The class II *pilE* TSS was mapped to nt "C" which is 8 nt downstream of the -10 box (Figure 4.5B) consistent with activity from the -10/-35 promoter.

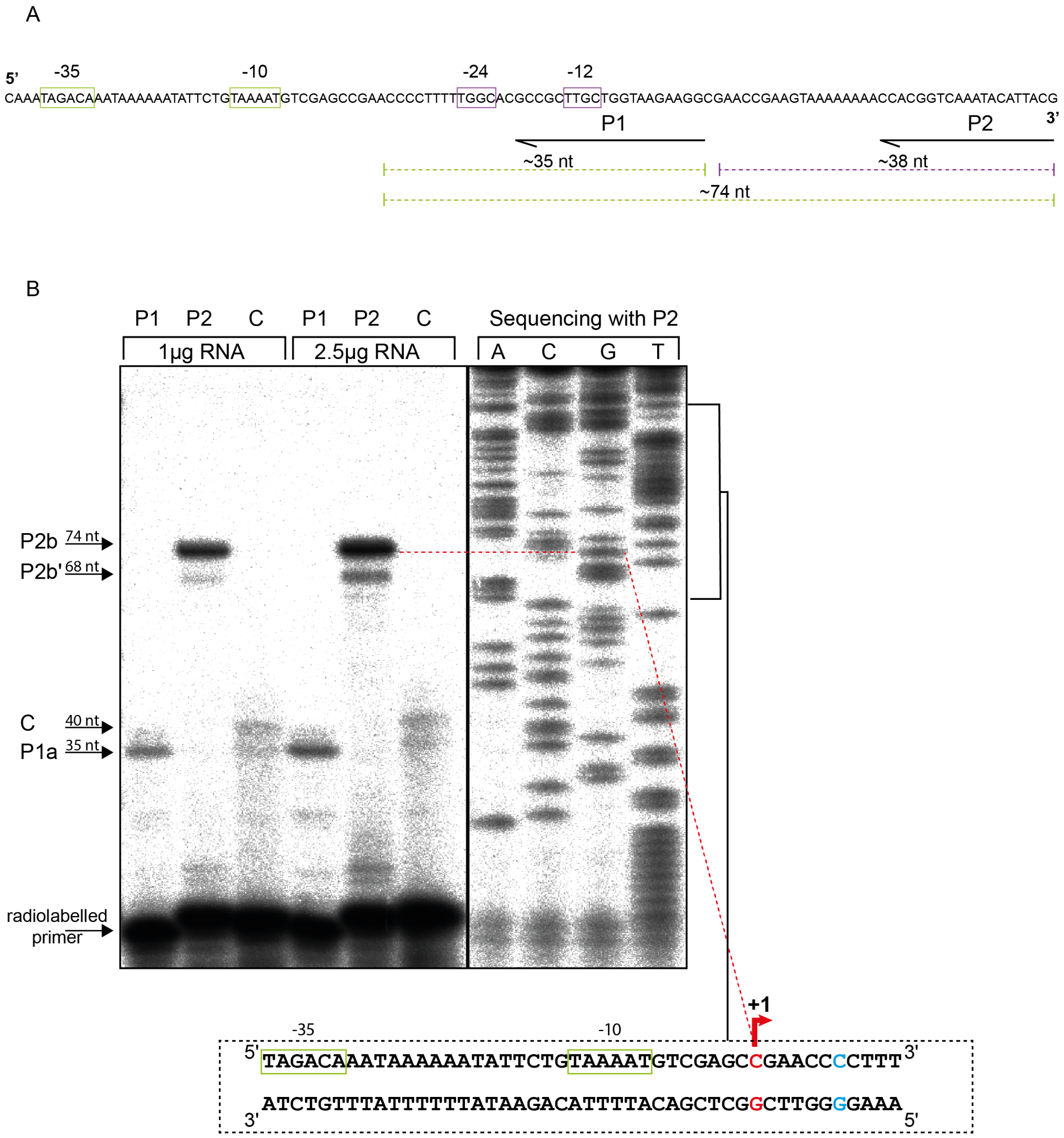


Figure 4.5 Identification of the class II *pilE* TSS

A. Region of class II *pilE* upstream sequence used to design primers for primer extension analysis. Arrows correspond to regions bound by P1 and P2. Putative promoter elements found upstream of the *pilE* start codon are boxed. B. Primer extension of 1 µg or 2.5 µg of RNA prepared from *N. meningitidis* S4 grown at 37^{o}C in liquid BHI to mid exponential phase. Arrows indicate product sizes generated with the control probe (C) and either P1 primer (P1a) or P2 primer (P2b and P2b'). Sequencing gel was run in parallel with P2 primer to determine the nucleotide position of the class II *pilE* TSS which is indicated by a red arrow. Nucleotide position shown in blue corresponds to P2b' product generated with P2 primer.

4.5 Class II *pilE* levels but not the TSS vary under different stress conditions

It has been reported previously that σ^N-dependent transcription requires environmental stimuli for upregulation of bEBPs. The experiments described this chapter so far were performed under standard laboratory conditions which may not mimic the conditions required for expression of σ^N or σ^N-dependent activators necessary for promoter activation. Therefore *N. meningitidis* S4 was subjected to a diverse panel of stress conditions, and *pilE* transcript levels as well as the TSS were examined. The panel of stresses chosen for the analysis was originally described for enteric bacteria (Christiansen *et al.*, 2004). Although the conditions do not necessarily have physiological relevance for *N. meningitidis*, these can provide insights into transcriptional responses in *N. meningitidis*. *N. meningitidis* S4 was grown at 37°C in liquid BHI, and when the OD_{600} of the cultures reached mid-exponential phase ($OD_{600} \approx 0.5$) bacteria were exposed to salt (5M NaCl), acid (HCl, pH 2.5), oxidative (0.15% H_2O_2) or envelope (5% ethanol or 5% Triton) stress for 10 min. In addition, S4 grown to the same OD_{600} but not exposed to any stress was used as a control (WT). RNA was collected and *pilE* transcript levels were assessed by Northern blot using a radiolabeled probe generated by amplifying 70 bp of *pilE* coding sequence. Interestingly, a number of conditions affected *pilE* transcript levels (Figure 4.6A). These include a) NaCl which downregulates *pilE* consistent with the previous findings in class I *pilE*-expressing *N. meningitidis* (Tan *et al.*, 2015), and b) pH which increased *pilE* transcript level. The effect of ethanol, Triton and H_2O_2 was difficult to assess because of the RNA degradation in samples as shown by Northern blot (Figure 4.6A). We also investigated whether *rpoN* transcript could be affected by the 10 min exposure to the stress stimuli. No *rpoN* transcript was detected by Northern blot analysis (Figure 4.6A) indicating that it is possible that *rpoN* mRNA levels are below the detection limit of this method, which is in line with the observation in Figure 4.1B showing low relative expression of *rpoN* as measured by qRT-PCR. Primer extension was also performed to detect whether any of the stress conditions affect the class II *pilE* TSS. Wild type *N. meningitidis* S4 (WT) exposed to no stress was used as a control, and primer P1 and P2 were used to generate the extension products. We detected products of ~35 nt with P1 (P1a) and ~74 nt with P2 (P2b), which are similar in length to those identified in Figure 4.5B. In addition to the ~68 nt product (P2b') which was detected previously with the primer P2 (Figure 4.5B), a smaller ~47 nt product (P2c) from bacteria exposed to Triton was detected (Figure 4.6B). Whether this is an RNA degradation product or an alternative TSS will be investigated in future. However we did not detect ~38 nt product with the primer P2, and there were no changes in primer extension products between WT and stress induced S4. Together the results suggest that *pilE* transcription is initiated from the σ^{70} -10/-35 promoter and there is no transcript initiated from the -12/-24 sequence under different environmental conditions (Figure 4.6B).

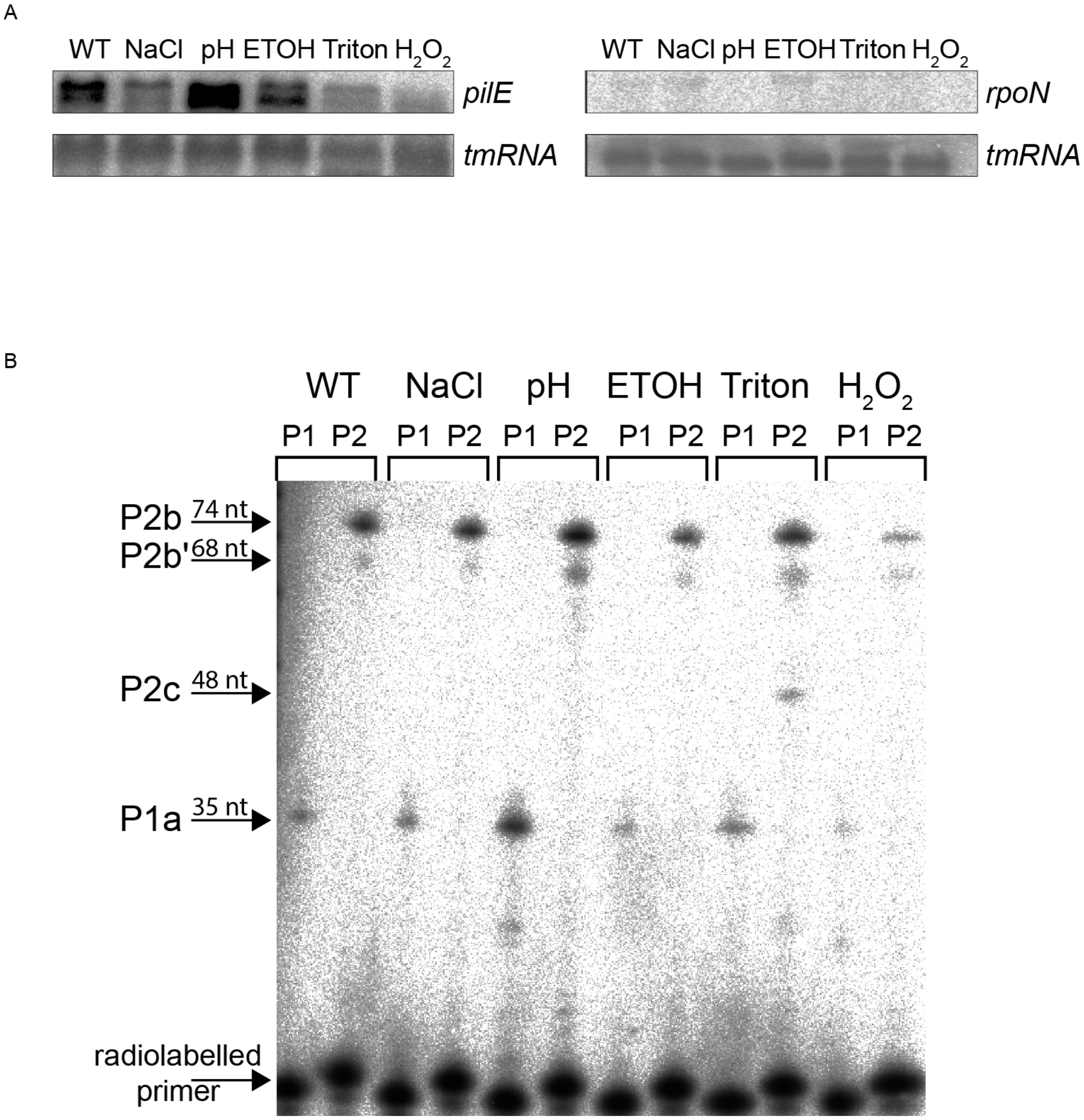


Figure 4.6 ***pilE*** **transcript levels in response to stress conditions**

A. RNA was collected from *N. meningitidis* S4 exposed to different stresses for 10 min, and was analysed by Northern blotting. Blot is representative of two biological repeats. B. Mapping TSS of *pilE* in bacteria subjected to different stress conditions using primers P1 and P2. No difference in class II *pilE* TSS was observed in bacteria exposed to no stress (WT) and those exposed to different stresses (shown). An additional primer extension product (P2c) was detected in Triton-treated samples. Experiment was performed on two independent occasions using two sets of biological replicates.

We considered the possibility that the low amount of *rpoN* transcript and the absence of detectable σ^N could result in the lack of σ^N-dependent transcription activation of class II *pilE*. Therefore we could not rule out that *rpoN* plays a role in *pilE* expression in specific conditions.

Therefore, as a final experiment we tested the effect of inducing *rpoN* expression on pilin levels. *N. meningitidis* S4 harbouring meningococcal C-terminal FLAG-tagged *rpoN* at the ectopic locus under IPTG-inducible promoter was constructed (S4P_{lac}*Nm* $\sigma^{N\text{-}FLAG}$). In parallel, we constructed *N. meningitidis* S4P_{lac}*Nel* $\sigma^{N\text{-}FLAG}$ strain containing IPTG-inducible *N. elongata rpoN*, which is known to be an activator of *pilE* transcription in *N. elongata* (Rendon *et al.*, 2013). Pilin levels in S4 overexpressing either *N. meningitidis* or *N. elongata rpoN* were analysed by Western blotting of cell lysates collected after overnight growth on solid medium supplemented with 1mM IPTG to induce *rpoN* expression. S4*ery* harbouring erythromycin resistance cassette at the same ectopic locus was used as a control. The expression of σ^N in S4P_{lac}*Nel* $\sigma^{N\text{-}FLAG}$ and S4P_{lac}*Nm* $\sigma^{N\text{-}FLAG}$ was confirmed with anti-FLAG antibody (Figure 4.7A). No difference in PilE protein levels were detected in S4P_{lac}*Nm* $\sigma^{N\text{-}FLAG}$ following *rpoN* expression compared to S4*ery* control. Since the expression of the *rpoN* in its native host does not affect class II pilin levels we conclude that meningococcal σ^N is unlikely to bind to *pilE* promoter, and therefore does not function as an activator of the *pilE* -12/-24 promoter in *N. meningitidis*.

Strikingly, the expression of *N. elongata rpoN* in *N. meningitidis* S4 led to reduction in PilE expression (Figure 4.7A). This was surprising as *N. elongata* has been reported to activate *pilE* transcription (Rendon *et al.*, 2013). *N. elongata* σ^N was shown to be functional in *N. elongata* grown to logarithmic phase (Rendon *et al.*, 2013) and in our experiment we tested *N. elongata* σ^N effect in overnight cultures which are in late stationary phase. Therefore to determine whether the effect of *N. elongata rpoN* overexpression observed in Figure 4.7A is growth phase dependent, S4P$_{lac}$*Nel* $\sigma^{N\text{-}FLAG}$ was grown in liquid BHI at 37^{o}C with or without IPTG and cell lysates were collected at different growth phases corresponding to 1, 3, 5 and 6 hrs following the σ^N induction. *N. elongata* σ^N expression was confirmed by Western blotting with anti-FLAG antibody and pilin expression was analysed with anti-pilin antibody (Figure 4.7B). In line with our previous observation, induction of *N. elongata* σ^N leads to a reduction in pilin expression compared to the isogenic control strain where no IPTG was added (Figure 4.7B), suggesting that under different growth phases *N. elongata* σ^N represses pilin expression in *N. meningitidis*. This interesting observation led us to speculate that when expressed in *N. meningitidis*, *N. elongata* σ^N can engage with the -12/-24 promoter upstream of class II *pilE* but is unable to initiate transcription possibly due to the absence of necessary bEBP in *N. meningitidis*, and this interaction of *N. elongata* σ^N with -12/-24 sequence blocks the transcription from the -10/-35 *pilE* promoter, hence decreasing pilin expression.

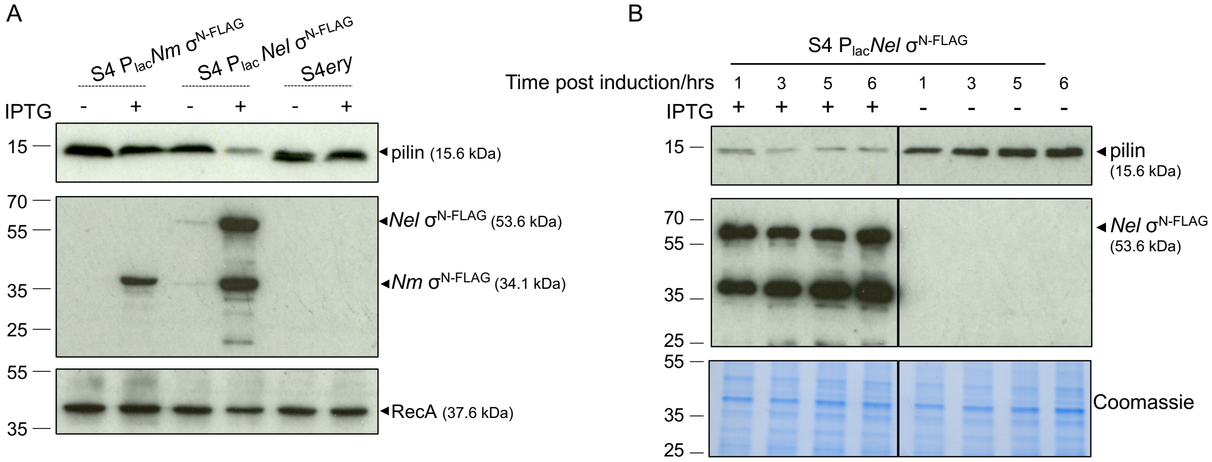


Figure 4.7 *N. elongata* σ^N reduces pilin expression in *N. meningitidis*

A. Western blotting showing pilin expression in *N. meningitidis* S4 expressing *N. elongata* σ^N or *N. meningitidis* σ^N after overnight growth on solid BHI at 37^{o}C. Expression of σ^N was checked using anti-FLAG antibody and RecA was used as a loading control. No change in pilin expression was detected in the presence of induced *N. meningitidis* σ^N whereas induction of *N. elongata* σ^N reduced pilin expression. B. S4P$_{lac}$*Nel* $\sigma^{N\text{-}FLAG}$ was grown in liquid BHI at 37^{o}C with or without IPTG (final concentration 1mM). Full length *N. elongata* σ^N is detected in the presence of IPTG. Expression of *N. elongata* σ^N leads to downregulation of class II pilin. Coomassie was used as a loading control.

Summary

In this chapter the role of meningococcal σ^N in class II *pilE* regulation was studied. We found that *N. meningitidis* σ^N does not function as a canonical σ^N-dependent activator when tested in *E. coli*. The lack of functional activity of meningococcal σ^N in *E. coli* could be attributed to the absence of *Neisseria* specific bEBP activators or additional -12/-24-dependent transcriptional elements such as IHF necessary for σ^N-dependent transcription. We therefore focused on understanding the function of *N. meningitidis* σ^N in *Neisseria* to eliminate differences that could arise by studies in a heterologous system.

rpoN transcript was detectable during *N. meningitidis* growth although the expression was low relative to the control tmRNA. However the absence of detectable σ^N protein suggests that *rpoN* is either not transcribed or the levels of σ^N are below the detection limit. The latter might well be correct because in *E. coli* the concentration of alternative σ factors is low compared to the housekeeping σ^D under standard laboratory conditions (Jishage *et al.*, 1996).

Earlier studies of *pilE* in pathogenic *Neisseria* showed that *N. meningitidis* class I *pilE* and *N. gonorrhoeae pilE* transcription relies the σ^{70}-dependent rather than the σ^N-dependent promoter. Our work shows that despite having a distinct promoter arrangement, class II *pilE* expression is similarly initiated from the σ^{70}-dependent promoter. Mapping the TSS of class II *pilE* further highlighted that σ^{70}-dependent transcription is responsible for class II *pilE* expression and validated the presence of the -10 element of the putative -10/-35 promoter located 8 nt away from the TSS. The absence of *rpoN* had no effect on pilin transcript levels and similarly overexpression of σ^N did resulted in any change in pilin protein levels, indicating that σ^N is not essential and has no implication for class II *pilE* regulation.

As each σ factor is associated with the response to a particular stress, the level of a given σ increases rapidly in the presence of an appropriate stimulus (Sharma *et al.*, 2010). Originally identified in the nitrogen starvation response, σ^N has since been implicated in the response to a diverse range of stimuli in numerous organisms (Buck *et al.*, 2000). Because there is no known meningococcal σ^N-specific stimulus, we tested a panel of extreme stress conditions to establish whether we could detect any change in *rpoN* (and *pilE*) transcript. Although changes in *pilE* levels in response to acid, salt and ethanol stress were recorded, these changes seem to be independent of σ^N as the TSS associated with the σ^{70}-dependent promoter remained unchanged under these conditions. Understanding the regulation of the class II *pilE* transcript in response to selective stimuli that lead to up- or downregulation of *pilE* merits further investigation, and may provide insights into the transcription factors involved in class II *pilE* modulation.

To bypass the need for a potential specific environmental stimuli for activation of σ^N, we designed a system where *N. meningitidis* S4 contained a copy of *rpoN* at an ectopic locus under an inducible promoter, allowing us to test class II pilin regulation when σ^N is present in excess. Comparison of pilin levels in the presence of induced *N. meningitidis* or *N. elongata* σ^N demonstrated that class II pilin levels are affected by *N. elongata* σ^N expression. However unlike in *N. elongata* where σ^N-dependent *pilE* activation is observed, class II PilE levels are reduced in *N. meningitidis* when *N. elongata* σ^N is expressed. This observation raises interesting questions as to how this effect is mediated. It remains to be determined whether reduction of class II pilin in the presence of *N. elongata* σ^N is a direct effect and whether it is taking place at the level of transcription. It is possible to address this by looking at the molecular mechanisms of *N. elongata* σ^N interaction with the meningococcal class II *pilE* promoter which harbours a mutated -12/-24 promoter sequence. It is not clear why the *rpoN* gene is conserved among *N. meningitidis* isolates and why we are able to detect *rpoN* transcription in *N. meningitidis*. One possible explanation is that σ^N in *N. meningitidis* has other roles and does not function as a canonical σ^N that binds consensus -12/-24.

In conclusion the results collectively demonstrate that class II *pilE* transcription is independent of σ^N and is initiated from -12/-35 promoter.

5 Results: Role of σ^{70} family members in class II *pilE* regulation

In the previous chapter we determined that σ^N does not regulate class II *pilE* suggesting that -12/-24 promoter upstream of *pilE* is not involved in pilin expression. Furthermore, our analysis of class II *pilE* promoter activity in *E. coli* together with the primer extension analysis revealed that the -10/-35 promoter upstream of class II *pilE* is responsible for pilin expression. Interestingly, some -10/-35 promoter sequences are able to recruit different σ factors belonging to σ^{70} family (Rhodius *et al.*, 2005; Wade *et al.*, 2006). For instance, the -10/-35 sequence upstream of σ^D-coding gene in *E. coli* can bind σ^D, σ^E and σ^S (Cho *et al.*, 2014). There are three σ^{70} members in *N. meningitidis* which have been identified based on their sequence similarity to orthologues found in *E. coli*: σ^D, σ^H and σ^E encoded by *rpoD*, *rpoH* and *rpoE* respectively (Heidrich *et al.*, 2017). Therefore, we aimed to investigate which if any σ^{70} family members present in *N. meningitidis* play a role in class II *pilE* expression.

Binding of multiple σ^{70} factor at the same -10/-35 site poses an interesting mechanism of gene regulation. If different σ^{70}-like factors bind the same promoter with different affinity, the strength of the promoter activation will vary, thus changing the gene expression (Huerta *et al.*, 2003; Rhodius *et al.*, 2010). Since members of the σ^{70} family are activated in response to specific stresses, binding of different σ^{70} could also ensure that a gene of interest is expressed under different conditions. Interestingly, promoters that can be recognized by more than one σ factor usually contain non-consensus -10/-35 sequences (Cho *et al.*, 2014; Lee *et al.*, 2001), which can in part explain their ability to bind different σ factors. For example, in *E. coli* the σ^S -10 recognition sequence is highly homologous to that of σ^D, whereas σ^S-dependent -35 sequence is degenerate (Typas *et al.*, 2007). This allows σ^S to bind both σ^S- and σ^D-dependent promoter sequences (Weber *et al.*, 2005).

Considering that class II *pilE* -10/-35 promoter contains near consensus -10 element but the putative -35 sequence shares low homology with the σ^D-dependent -35 consensus sequence, we hypothesised that class II *pilE* promoter may be regulated by another, or multiple σ^{70}-like factors. Since little is known about the σ^{70} family members in *N. meningitidis*, we first determined the presence and the conservation of σ^{70} family members in *N. meningitidis* during bacterial growth, then examined pilin levels in mutants lacking or overexpressing different σ^{70} factors.

5.1 Bioinformatic analysis of σ^{70} family members in *N. meningitidis*

To date three σ^{70} family members have been identified in several strains of *N. meningitidis*. Therefore, we first aimed to determine the presence and the conservation of the three σ^{70} family members (σ^D, σ^E and σ^H) in *N. meningitidis*. We analysed the genomes of 290 meningococcal strains (same dataset as described in Chapter 3) and compared the domain organisation of the deduced σ factor sequences with homologues in *E. coli*. As an extension of this analysis, we also determined the presence and conservation of σ^{70} family coding genes in the publicly available genomes of other *Neisseria* species: two strains of *N. gonorrhoeae* (FA1090, MS11), and five commensal species including *N. cinerea* (14685), *N. lactamica* (23970), *N. elongata* (29315), *N. mucosa* (25996) and *N. subflava* (9703).

σ^D. Using full length *rpoD* sequence from *N. meningitidis* 8013 and BLAST analysis, *rpoD* homologs were identified in all 290 meningococcal isolates. The sequences were extracted, translated and aligned using CLUSTALW. σ^D was found to be highly conserved, exhibiting >99% amino acid sequence identity among the 290 meningococcal isolates (Figure 5.1A). There was no association between a specific σ^D sequence and class I or class II *pilE*. The comparison of the predicted σ^D protein sequences between *N. meningitidis* 8013 and other *Neisseria* species also revealed that the sequence of this protein is highly conserved in all *Neisseria* spp. (Figure 5.1A).

Protein domain composition was assessed by comparing the deduced meningococcal σ sequence to the known *E. coli* σ^D (Severinova *et al.*, 1996). *N. meningitidis* σ^D shares 54% amino acid sequence identity with *E. coli* σ^D (Figure 5.1B). *E. coli* σ^D contains four regions (Region 1-4) each associated with a specific function (discussed in section 1.5.1.1). The DNA binding motifs are located in Region 2.4 and 4.2, which mediate the recognition of -10 and -35 promoter elements respectively. Region 2.3 contains a signature RpoD box domain which is also involved in mediating interactions with the DNA (Gopal *et al.*, 1997). Comparison of the σ^D domains demonstrated that Region 4.2, 2.4 and RpoD box are almost identical between *E. coli* and *N. meningitidis*, suggesting that σ^D interaction with -10/-35 promoter might be similar in these species (Figure 5.1B). This observation is interesting considering that *N. meningitidis* genome lacks the conserved -35 element of the -10/-35 promoters (Heidrich *et al.*, 2017). *E. coli* σ^D also contains the non-conserved region (NCR) positioned between Region 1.2 and Region 2.1 (Figure 5.2B) (Kumar *et al.*, 1995). NCR, identified in only a few housekeeping σ factors, is the most diverse region of σ^D, which is known to contribute to σ^{70} binding to RNAP (Leibman *et al.*, 2007). The NCR alignment between *E. coli* and *N. meningitidis* revealed low sequence identity (24%) suggesting that the two species might interact with the cognate RNAP differently.

A

Species	Conservation with 8013 σ^D
N. meningitidis class I *pilE* 213 strains	>99
N. meningitidis class II *pilE* 77 strains	>99
N. gonorrhoeae FA1090	99
N. gonorrhoeae MS11	96
N. cinerea	99
N. lactamica 23970	99
N. elongata 29315	82
N. mucosa	91
N. subflava	89

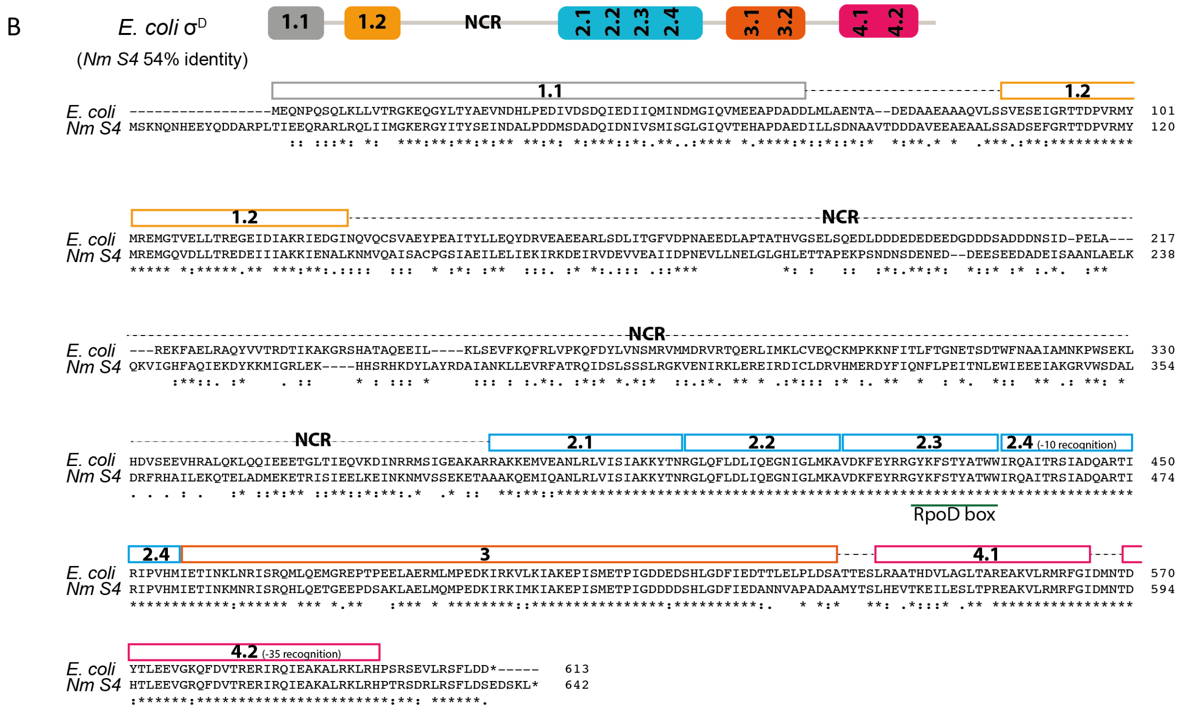


Figure 5.1 Bioinformatic analysis of meningococcal σ^D

A. Percentage identity of σ^D in class I and class II *pilE* expressing *N. meningitidis* and comparison of the deduced σ^D amino acid sequences in other *Neisseria* species to the reference σ^D sequence from 8013. B. Comparison of σ^D domains between *E. coli* and *N. meningitidis* S4 (*Nm*) σ^D. *N. meningitidis* σ^D shares 54% identity with *E. coli* σ^D. Colour-coded bars above the sequence represent the conserved regions of *E. coli* σ^D: 1.1, grey; 1.2 pale orange, NCR non-conserved domain; 2.1-2.4, blue; 3, dark orange; 4.1 and 4.2, red. Schematic of domain organisation found in *E. coli* is shown above the alignment.

$\boldsymbol{\sigma^E}$ belongs to the Group 4 extracytoplasmic factors (ECF) which are structurally the most diverse group of σ^{70} proteins. Approximately 94 different groups of ECFs have been identified in bacteria and the difference between the groups lies in a) their amino acid sequences, b) the presence of an anti-σ factor, c) the genomic locus and d) the σ-dependent promoter elements associated with the ECF (Anna *et al.*, 2009). *E. coli* σ^E is a member of ECF02 group and is characterised by two domains: 1) Region 2 which is responsible for RNA polymerase and promoter recognition and 2) Region 4 that mediates interaction with a cognate anti-σ factor (RseA), which are the most well characterised regulators of ECF activity (Tam *et al.*, 2002). *E. coli* anti-σ^E contains two modules: 1) N-terminal anti-sigma domain (ASD) that blocks σ^E activity and 2) membrane-bound C terminus that allows anti-σ^E to sense the extracytoplasmic stress signals and release σ^E for transcription initiation (Campbell *et al.*, 2007).

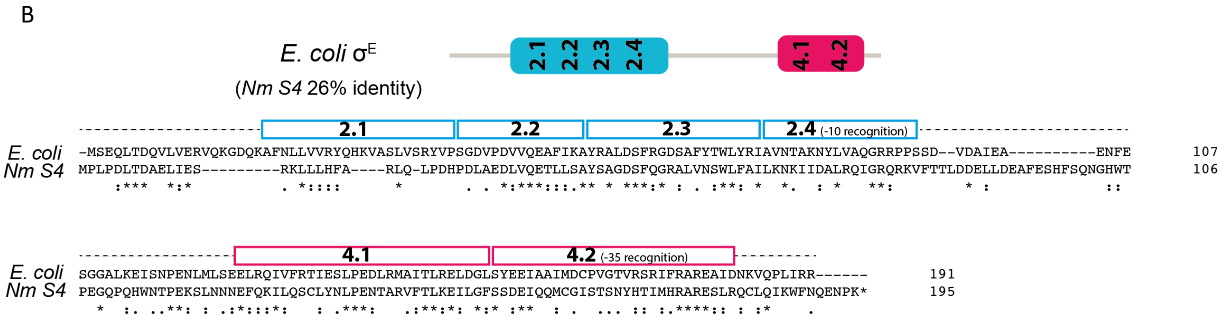
N. meningitidis σ^E belongs to ECF13 group (Anna *et al.*, 2009). *N. gonorrhoeae* and *N. meningitidis* σ^E are the only two members of ECF13 for which functional data is available (Gunesekere *et al.*, 2006a; Hopman *et al.*, 2010). Unlike *E. coli*, meningococcal anti-σ^E contains a soluble C-terminal domain suggesting it functions as a cytoplasmic, rather than a membrane sensor. Indeed in *N. gonorrhoeae* σ^E has been reported to respond to changes in the cellular environment for example during oxidative stress (Gunesekere *et al.*, 2006). Meningococcal anti-σ^E is also predicted to have ASD domain which contains a zinc-containing anti-σ factor (ZAS) domain that is absent in *E. coli* anti-σ^E (Hopman *et al.*, 2010).

Similar to σ^D analysis, we used the full-length sequence of *N. meningitidis* 8013 σ^E and anti-σ^E to conduct BLAST searches of meningococcal genomes containing either class I or class II *pilE*. Results, as shown in Figure 5.2A, demonstrate that both class I and class II *pilE* carrying isolates have σ^E homologues, and amino acid identity of σ^E and anti-σ^E among all isolates is near identical irrespective of which class of *pilE* they have. Comparison of other *Neisseria* σ^E/anti-σ^E sequences revealed that both *N. gonorrhoeae* and *N. lactamica* σ^E display high sequence identity (>90%) with *N. meningitidis* 8013 anti-σ^E / σ^E (Figure 5.2A). However lower sequence homology was observed between 8013 anti-σ^E / σ^E and several other commensal species including *N. mucosa* (51% / 73%), *N. subflava* (46% / 73%/) and *N. elongata* (43% / 41%/). Interestingly, the ASD of anti-σ^E is conserved among *Neisseria* species (Hopman *et al.*, 2010) implying that they may interact with their cognate σ^E in the same manner. The lack of conservation in the anti-σ^E C-terminal region in some pathogenic and commensal *Neisseria* may suggest that there are differences in the mechanisms of regulation of σ^E and possibly variation in the stress stimuli that anti-σ^E respond to.

Comparison of the σ^E domain organisation between *E. coli* and *N. meningitidis* highlighted differences between the two species (Figure 5.2B). *E. coli* σ^E shares 26% amino acid similarity with meningococcal σ^E and 26% identity with anti-σ^E, in line with the observation that two σ factors belong to two distinct ECF groups. Furthermore there was little homology between the DNA binding recognition domains of *E. coli* and *N. meningitidis* σ^E (region 2.4 and 4.2) (Figure 5.2B). Thus activity and regulation of these two σ^E might be different.

A

Species	Conservation with 8013	
	σ^E	anti-σ^E
N. meningitidis class I *pilE* 213 strains	>99	>99
N. meningitidis class II *pilE* 77 strains	>99	>99
N. gonorrhoeae FA1090	98	95
N. gonorrhoeae MS11	98	95
N. cinerea	94	90
N. lactamica 23970	97	93
N. elongata 29315	41	43
N. mucosa	73	51
N. subflava	73	46



```
E. coli -MSEQLTDQVLVERVQKGDQKAFNLLVVRYQHKVASLVSRYVPSGDVPDVVQEAFIKAYRALDSFRGDSAFYTWLYRIAVNTAKNYLVAQGRRPPSSD--VDAIEA----------ENFE    107
Nm S4   MPLPDLTDAELIES---------RKLLLHFA----RLQ-LPDHPDLAEDLVQETLLSAYSAGDSFQGRALVNSWLFAILKNKIIDALRQIGRQRKVFTTLDDELLDEAFESHFSQNGHWT    106
            :***  *:*           . *::::       *        . . *:***:::.** * ***:* : . :**: *  *.  : *   **:         * :             ::

E. coli SGGALKEISNPENLMLSEELRQIVFRTIESLPEDLRMAITLRELDGLSYEEIAAIMDCPVGTVRSRIFRAREAIDNKVQPLIRR------    191
Nm S4   PEGQPQHWNTPEKSLNNNEFQKILQSCLYNLPENTARVFTLKEILGFSSDEIQQMCGISTSNYHTIMHRARESLRQCLQIKWFNQENPK*    195
          *  :. ..**: : .:*:::*:   : .***:   .:**:*: *:* :**  : .  ... :: :.****:: : :*     .
```

Figure 5.2 Bioinformatic analysis of meningococcal σ^E

A. Percentage identity of σ^E and anti-σ^E in *Neisseria* species compared to the deduced σ^E sequence from *N. meningitidis* 8013. B. Alignment of amino acid sequences between *E. coli* and *N. meningitidis* (*Nm*) S4 σ^E and sequence identity between the two σ^E (26%). *E. coli* σ^E domain boxes shown above. Domains are colour coded (see Figure 5.1B)

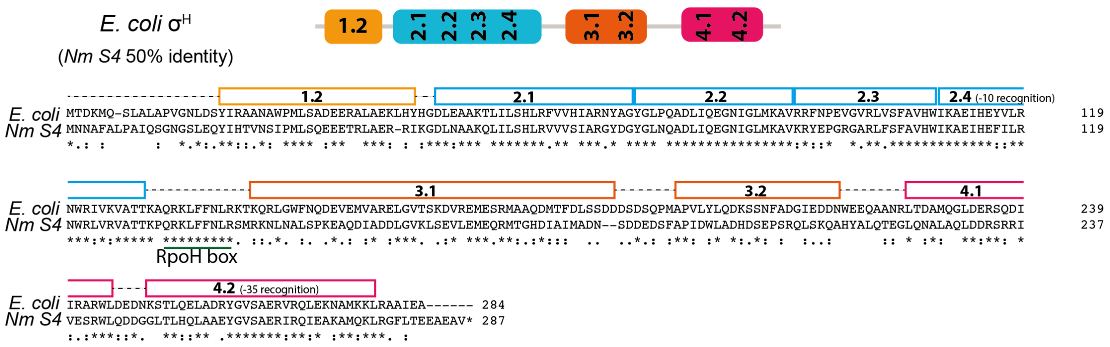
σ^H. The σ^H encoded by *rpoH* from *N. meningitidis* 8013 (Heidrich *et al.*, 2017) was used to perform BLAST searches of meningococcal isolates. *rpoH* was present in all *N. meningitidis* genomes analysed. The deduced meningococcal σ^H sequences displayed high amino acid conservation, and similar to σ^{70} and σ^E, there was no correlation between σ^H sequence and pilin class. Amino acid sequence comparison of *N. meningitidis* 8013 σ^H and other *Neisseria* species showed high sequence similarity among the *Neisseria* (>83%). (Figure 5.3A)

N. meningitidis σ^H shares 50% amino acid sequence identity with *E. coli* σ^H. Alignment of the known *E. coli* σ^H domains with *N. meningitidis* σ^H demonstrated that several key DNA binding sites exhibit near identical sequence (Figure 5.3B). Meningococcal σ^H contains the characteristic RpoH box, present in other bacterial σ^H (Nakahigashi *et al.*, 1995) which plays a role in σ^H interaction with RNAP (Arsène *et al.*, 1999). Furthermore, Region 2.4 which mediates -10 promoter recognition in *E. coli* shows 81% amino acid sequence identity between *N. meningitidis* and *E. coli*. Region 4.2 which is involved in -35 promoter recognition in *E. coli* displayed 62% amino acid sequence similarity with the meningococcal σ^H. The high sequence identity in the promoter recognition domains of *E. coli* and *N. meningitidis* σ^H suggests that both σ^H may recognise similar -10/-35 promoter elements. This is interesting considering that no σ^H-dependent promoters have been identified in *N. meningitidis* (Heidrich *et al.*, 2017) and putative σ^H-dependent promoters annotated in *N. gonorrhoeae* are distinct from the known *E. coli* σ^H-dependent -10/-35 sequence (Gunesekere *et al.*, 2006).

A

Species	Conservation with 8013 σ^H
N. meningitidis class I *pilE* 213 strains	>99
N. meningitidis class II *pilE* 77 strains	>99
N. gonorrhoeae FA1090	99
N. gonorrhoeae MS11	99
N. cinerea	97
N. lactamica 23970	98
N. elongata 29315	83
N. mucosa	95
N. subflava	94

B



```
E. coli MTDKMQ-SLALAPVGNLDSYIRAANAWPMLSADEERALAEKLHYHGDLEAAKTLILSHLRFVVHIARNYAGYGLPQADLIQEGNIGLMKAVRRFNPEVGVRLVSFAVHWIKAEIHEYVLR    119
Nm S4   MNNAFALPAIQSGNGSLEQYIHTVNSIPMLSQEEETRLAER-RIKGDLNAAKQLILSHLRVVVSIARGYDGYGLNQADLIQEGNIGLMKAVKRYEPGRGARLFSFAVHWIKAEIHEFILR    119
        *.: :      :  *.*:.**::.*: **** :**  ***: : :***:*** *******.** ***.* **** ****************:*::*  *.**.*************::**

E. coli NWRIVKVATTKAQRKLFFNLRKTKQRLGWFNQDEVEMVARELGVTSKDVREMESRMAAQDMTFDLSSDDDSDSQPMAPVLYLQDKSSNFADGIEDDNWEEQAANRLTDAMQGLDERSQDI    239
Nm S4   NWRLVRVATTKPQRKLFFNLRSMRKNLNALSPKEAQDIADDLGVKLSEVLEMEQRMTGHDIAIMADN--SDDEDSFAPIDWLADHDSEPSRQLSKQAHYALQTEGLQNALAQLDDRSRRI    237
        ***:*:***** *********. ::.*. :. .*.: :* :***. .:* ***.**:.:*:::  ..  ..*.: :**: :* *:.*: :  :..:      :: * :*:  **:**: *

E. coli IRARWLDEDNKSTLQELADRYGVSAERVRQLEKNAMKKLRAAIEA------ 284
Nm S4   VESRWLQDDGGLTLHQLAAEYGVSAERIRQIEAKAMQKLRGFLTEEAEAV* 287
        :.:***::*.  **::** .*******:**:* :**:***. :
```

Figure 5.3 Bioinformatic analysis of meningococcal σ^H

A. σ^H amino acid identity between class I and class II *pilE* expressing *N. meningitidis* and in *Neisseria* species compared to the deduced σ^H sequence from *N. meningitidis* 8013. B. CLUSTALW alignment of σ^H sequences between *E. coli* and *N. meningitidis* S4 (*Nm*). *E. coli* σ^H shares 50% amino acid identity with *N. meningitidis* σ^H. *E. coli* σ^H domain boxes are shown above the alignments and colour coded (see Figure 5.1B).

5.2 Levels of different σ factors during *N. meningitidis* growth

The presence of conserved genes encoding σ^D, σ^H and σ^E factors in *N. meningitidis* led us to determine the expression levels of three σ factors in S4. We examined transcript and protein levels of σ^{70}-like factors under steady state during bacterial growth.

To measure *rpoD*, *rpoE* and *rpoH* expression WT S4 was grown in BHI liquid at 37°C for 22 hours and RNA samples were collected at 3, 6, 8 and 22 hours during the growth. Each time point represents a bacterial growth phase thus allowing us to monitor any growth phase-dependent changes in expression of the σ factors. Relative transcript levels were measured using qRT-PCR and the experiment was performed using three independent biological repeats. All three σ factors were expressed throughout bacterial growth. However, the relative expression levels varied among the three σ^{70} members (Figure 5.4). Interestingly, *rpoH* transcript was the most abundant among the three σ factors (Figure 5.4C) and *rpoE* transcript levels were the lowest relative to the standard control tmRNA (Figure 5.4B), which is consistent with previous reports stating that during mid-log phase the meningococcal *rpoE* transcript is almost undetectable (Hopman *et al.*, 2010). The relative transcript levels of all three σ factors remained unchanged throughout the growth (Figure 5.4). This is in contrast to other bacterial species including *E. coli* where *rpoD*, *rpoE* and *rpoH* growth phase-dependent expression has been observed (Fujita *et al.*, 1994; Klein *et al.*, 2016; Wagner *et al.*, 2009).

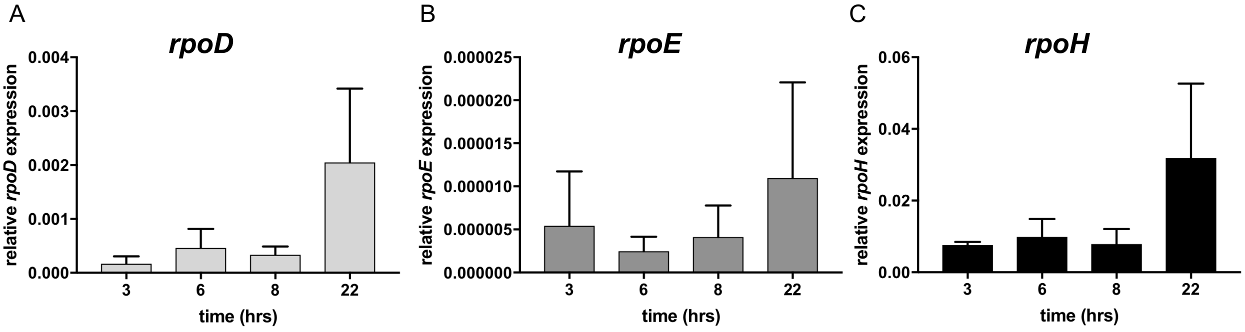


Figure 5.4 Relative expression levels of three σ^{70} family factors in *N. meningitidis*

Strand-specific qRT-PCR analysis of relative A. *rpoD*, B. *rpoE* and C. *rpoH* levels in S4 at different time points during the growth (3, 6, 8 and 12 hrs). Amount of transcript is represented as R value standardised to tmRNA transcript levels. Error bars represent standard deviation and the experiment was performed three times using three independent replicates analysed in triplicate. No statistical difference in the relative expression of A. *rpoD*, B. *rpoE* or C. *rpoH* transcript was detected during the growth (One-way ANOVA).

Given that σ factors transcripts were detected, we next examined protein levels during bacterial growth. In order to determine σ factor expression, S4 strains expressing C terminal FLAG-tagged σ factors were constructed. Attempts to make S4 carrying FLAG tagged σ^D were unsuccessful therefore we were unable to study σ^D expression during meningococcal growth. S4$\sigma^{H\text{-FLAG}}$ was constructed successfully and cell lysates were collected during bacterial growth for 22 hours in BHI liquid. σ^H protein levels were examined at 3, 6, 8 and 22 hours which corresponded to early-, mid-, late-logarithmic and stationary phases. σ^H was detectable throughout bacterial growth and the expression was upregulated during mid-, late-logarithmic and stationary phases (6, 8 and 22 hrs respectively) compared to early-logarithmic phase (3 hrs) (Figure 5.5A), based on one biological repeat. Experiments are currently underway to perform additional repeats and quantifications based on the loading control RecA expression levels. Furthermore, S4$\sigma^{E\text{-FLAG}}$ has been constructed and was grown overnight on solid BHI medium and cell lysates were collected to measure σ^E expression. σ^E was detected after overnight growth (Figure 5.5B). We are in the process of examining the expression of σ^E during bacterial growth in order to conduct the comparison of the expression levels of the σ^H and σ^E in *N. meningitidis*.

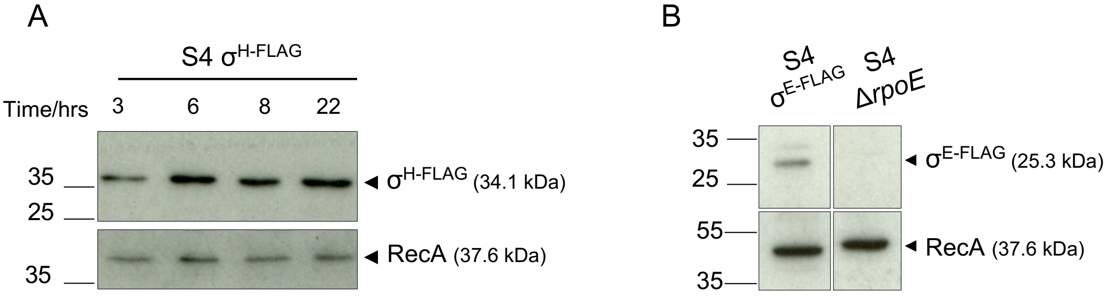


Figure 5.5 σ^H and σ^E expression in *N. meningitidis*

A. S4 containing FLAG-tagged endogenous *rpoH* was grown in liquid BHI at 37°C for 22 hrs. Full length σ^H was detected using anti-FLAG antibody and RecA was used as a loading control. σ^H was present throughout bacterial growth (n=1). B. Western blot analysis of σ^E levels in whole cells lysates collected from S4$\sigma^{E\text{-}FLAG}$ grown overnight on solid BHI medium, using anti-FLAG antibody. S4 lacking *rpoE* (S4Δ*rpoE*) was used as a negative control and RecA was used as a loading control (n=1).

5.3 Pilin levels in σ^{70} family mutants

The number of RNAP molecules within the cell is limited therefore a successful formation of the RNAP-σ factor complex depends on the concentration of free σ molecules and their affinity for RNAP (Österberg *et al.*, 2011). Previous studies in *E. coli* indicate that the level of RNAP does not vary throughout bacterial growth whereas the concentration of σ factors can change in response to different stresses (Jishage *et al.*, 1996) and changes in the level of σ factors lead to activation of specific σ-dependent promoters. The information about σ factor regulation in *N. meningitidis* is very limited. Meningococcal σ^E regulon has been shown to be comprised of 11 genes but did not include *pilE*, although the analysis was performed in class I *pilE* expressing *N. meningitidis* H44/76 (Huis in 't Veld *et al.*, 2011). σ^H has been identified in a closely related pathogen *N. gonorrhoeae* (Du *et al.*, 2005; Laskos *et al.*, 2004) but σ^H regulon has not been described in *N. meningitidis*. σ^D has been annotated as the housekeeping σ factor although there has been no functional analysis of meningococcal σ^D. Therefore, we hypothesised that if the *pilE* -10/-35 promoter can bind different σ^{70} members, changing the levels of one of the σ^{70}-could impact pilin expression. Therefore we constructed *N. meningitidis* mutants either lacking or overexpressing the σ^{70} family factors and examined pilin protein levels in these backgrounds.

σ^D. Housekeeping σ^{70} factor σ^D is essential in *E. coli*. Based on the analysis of essential genes in *N. meningitidis* 8013 and *N. gonorrhoeae* FA1090, *rpoD* is also essential in pathogenic *Neisseria* species (Capel *et al.*, 2016; Remmele *et al.*, 2014). We attempted to perform ORF replacement of *rpoD* with a kanamycin cassette in *N. meningitidis* S4, but this was unsuccessful. Interestingly, however, a transposon (Tn) insertion mutant in *rpoD* of *N. meningitidis* strain 8013 constructed by V. Pelicic was viable and had no phenotypic growth defect when grown on solid media (unpublished). The Tn insertion was mapped between Region 1 and Region 2 of σ^{70}, which corresponds to a putative non-conserved region (NCR) based on the alignment with *E. coli* σ^{70}. The NCR is present in the majority of the *rpoD* factors of Gram-negative bacteria although there are some exceptions (Vingadassalom *et al.*, 2005). Deletion of NCR of σ^{70} in *E. coli* leads to σ^{70}-dependent elongation pausing as σ^{70} fails to leave the promoter and is followed by impaired growth (Leibman & Hochschild, 2007). To examine whether pilin expression is affected by the presence of Tn insertion within the putative NCR of *N. meningitidis rpoD*, the Tn insertion was introduced into *N. meningitidis* S4 using PCR amplified *rpoD*:Tn locus from 8013. Three single colonies generated from three independent transformations were collected and the *rpoD* locus was checked by PCR for the presence of Tn insertion. Three single colonies of S4*rpoD*::Tn were grown overnight, and cell lysates were collected to determine pilin expression by Western blotting. No changes in pilin expression were detected in *rpoD*:Tn background compared to WT S4 control suggesting that under the condition tested the disruption of *rpoD* at the native locus by Tn insertion does not affect pilin expression (Figure 5.6A). However, at present it is not clear whether the Tn insertion within the NCR affects the folding or functional characteristics of the meningococcal σ^{70}.

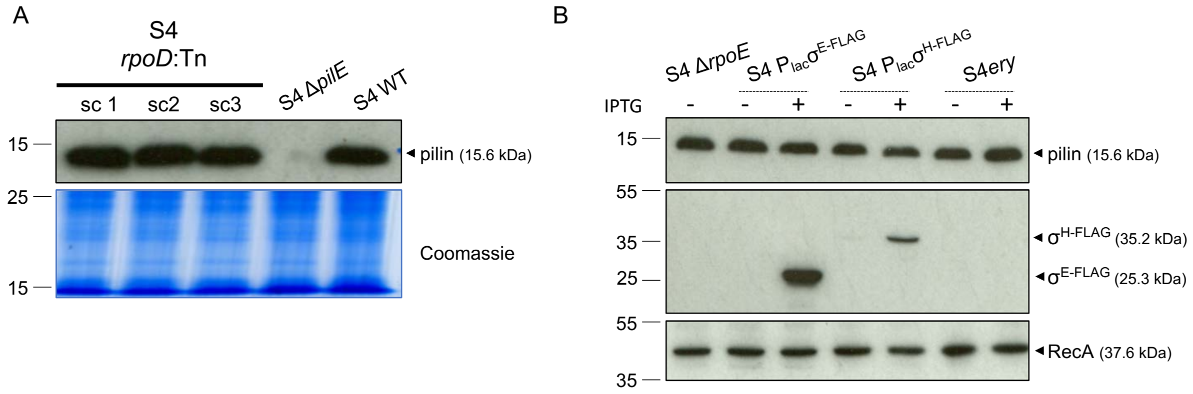


Figure 5.6 Pilin levels in σ factor mutants

Western blotting analysis of pilin levels in whole cell lysates collected from the following strains grown overnight on solid BHI medium at 37°C: A. three independent single colonies (sc 1-3) of S4*rpoD*::Tn and two control strains S4 Δ*pilE* and S4 wild type (WT), and B. S4 Δ*rpoE*, S4$_{\mathrm{Plac}}\sigma^{\mathrm{E\text{-}FLAG}}$, S4 $\mathrm{P}_{\mathrm{lac}}\sigma^{\mathrm{H\text{-}FLAG}}$ and control strain S4*ery*. The expression of IPTG-induced σ^{E} and σ^{H} (1mM IPTG final concentration) was examined using anti-FLAG antibody. Coomassie (A) and RecA (B) were used as a loading controls and each blot is a representative of two independent experiments.

σ^{H} has been shown to be essential in *N. meningitidis* and *N. gonorrhoeae* (Capel *et al.*, 2016; Laskos *et al.*, 2004). Our attempts to construct a S4Δ*rpoH* strain were also unsuccessful. Therefore, to address whether σ^{H} plays a role in class II pilin expression regulation, we measured pilin levels in the presence of induced σ^H expression. S4P$_{lac}\sigma^{H\text{-}FLAG}$ was constructed harbouring a C terminal FLAG tagged *rpoH* under IPTG-inducible *lac* promoter at the ectopic locus (between *trpB* and *igA* genes). S4P$_{lac}\sigma^{H\text{-}FLAG}$ was grown overnight and σ^H expression was induced by addition of 1mM IPTG. In the presence of induced σ^H, pilin levels were comparable to WT S4 (Figure 5.6B). No difference in pilin expression was observed between S4P$_{lac}\sigma^{H\text{-}FLAG}$ and an isogenic S4P$_{lac}\sigma^H$ harbouring non-tagged *rpoH*, suggesting that FLAG tag does not interfere with σ^H function (data not shown). We can conclude that induced expression of σ^H does not affect pilin levels under the condition tested.

σ^E. Unlike σ^D and σ^H, meningococcal σ^E is not essential (Huis in 't Veld *et al.*, 2011). Therefore we were able to generate S4Δ*rpoE* by replacing the ORF of *rpoE* with an erythromycin resistance cassette. Based on the analysis of σ^E in *N. meningitidis*, *rpoE* is arranged in an operon of six genes (Heidrich *et al.*, 2017). Immediately downstream of *rpoE* there is a gene encoding anti-*rpoE* and under steady state the expression of the operon is relatively low. However, deletion of anti-*rpoE* leads to upregulation of *rpoE* (Huis in 't Veld *et al.*, 2011). Low basal σ^E expression can mask any potential effect of this σ factor, therefore S4 strain overexpressing *rpoE* was constructed by inserting *rpoE* with or without a C-terminal FLAG tag at the ectopic locus under the control of a *lac* promoter, resulting in S4P$_{lac}\sigma^{E\text{-}FLAG}$ and S4P$_{lac}\sigma^E$ respectively. The strains were grown overnight on solid BHI and *rpoE* expression was induced by addition of 1mM IPTG. Pilin levels were examined in the presence of induced $\sigma^{E\text{-}FLAG}$ and in σ^E negative background (Figure 5.6B). Pilin levels remained unaffected in the presence of induced $\sigma^{E\text{-}FLAG}$ or in $\Delta\sigma^E$ background (Figure 5.6B). To exclude the possibility that no changes in pilin expression are observed because FLAG tag obstructs the function of σ^E, we examined pilin levels in S4P$_{lac}\sigma^E$ following σ^E induction.

No difference in pilin protein levels were detected in S4$P_{lac}\sigma^{E}$ compared to S4$P_{lac}\sigma^{E\text{-FLAG}}$ or S4*ery* (data not shown). This implies that σ^{E} is not essential for pilin production.

Summary

N. meningitidis contains three σ^{70} family factors and our analysis demonstrates that the sequences of these σ factors are conserved across meningococcal isolates and other *Neisseria* species, with the exception of the σ^{E}/anti-σ^{E} which is different in *N. elongata*. Furthermore, the expression of *rpoD*, *rpoE* and *rpoH* was detected throughout bacterial growth and no growth phase-dependent transcriptional regulation was observed for any of the σ factors. It would be interesting to address whether any stimuli such as nutrient limitation, acid or temperature stress which have been shown to regulate σ factor transcription in *E. coli* (Durfee *et al.*, 2008; King *et al.*, 2010; Tilly *et al.*, 1986) could affect the transcription of meningococcal σ factors, as this will provide insight into the mechanisms of σ factor regulation in *N. meningitidis*. In addition, quantitative Western blotting can be performed to measure the relative levels of each σ factor and determine how the σ factor protein levels correlate with the corresponding transcript levels during bacterial growth.

The comparison of promoter recognition domains of σ^{D} and σ^{H} between *E. coli* and *N. meningitidis* revealed high (>90%) sequence identity implying that -10/-35 association with -10/-35 sequence might also be similar between the two species. This is interesting considering that *E. coli* and *N. meningitidis* σ^{70}-recognition site sequences are different. The majority of *N. meningitidis* genes contain conserved consensus -10 promoter element and only 7% of these genes are predicted to have canonical -35 σ^{70}-dependent sequences

(Heidrich *et al.*, 2017). Furthermore, in contrast to *E. coli*, global promoter analysis did not identify any σ^{H}-dependent promoter consensus sequences in *N. meningitidis* or *N. gonorrhoeae* (Heidrich *et al.*, 2017; Remmele *et al.*, 2014). Thus the absence of distinct promoter sequences for σ^{H} and σ^{D} suggests that in *N. meningitidis* -10/-35 promoter sequences can be recognised by multiple σ^{70} members, which offers an additional regulatory mechanism for *N. meningitidis* to control the expression of genes. Experiments to test this prediction will include ChIP-seq using the tagged σ^{70} factors, which will provide a map of σ factor recognition sequences across meningococcal genome.

The possibility that multiple σ factors can recognise the same -10/-35 sequence lead us to consider whether the class II *pilE* gene, which contains a consensus -10 and degenerate -35 could be regulated by any of the σ^{70} members found in *N. meningitidis*. In line with this hypothesis, the class I *pilE* promoter, which has long been thought to be regulated by σ^{D} has recently been bioinformatically annotated as σ^{E} promoter (Heidrich *et al.*, 2017). This annotation was based on the sequence homology between putative class I *pilE* -35 box and *E. coli* σ^{E} -35 consensus sequence however no experimental analysis was performed to validate promoter annotation, and earlier studies showed that class I *pilE* was not part of meningococcal σ^{E} regulon (Huis in 't Veld *et al.*, 2011). Nevertheless, it supports the hypothesis that at least class I *pilE* promoter could be recognized by both σ^{E} and σ^{D}. Our analysis of class II pilin levels revealed that σ^{E} is expressed but it is not essential for pilin expression under the growth conditions tested. Furthermore, the overall conclusion that emerged from these experiments is that none of the σ^{70} factor mutants tested here result in changes in pilin expression. There are several possible explanations as to why pilin remains unchanged in these mutants. One possibility is that the *pilE* -10/-35 promoter is regulated by only housekeeping σ^D and does not bind any other σ factors. We attempted to prove this using S4 strains with Tn insertion at the *rpoD* (Figure 5.6A). However at present we are uncertain whether Tn insertion in NCR of σ^D affects σ^D promoter binding and function and therefore we cannot draw meaningful conclusion from this experiment (Figure 5.6A). Deletion of NCR in *E. coli* σ^D was reported to induce severe growth defects (Leibman & Hochschild, 2007). Thus it is unlikely that Tn insertion in meningococcal *rpoD* had any impact on its activity, since it did not result in any growth defective phenotype. Further work is necessary to validate the proposed meningococcal σ^D domain organisation, and to characterise σ^D-dependent promoter recognition in *N. meningitidis*.

In this work we did not quantify levels of σ factors therefore we cannot compare the relative expression of these factors following the induction of σ^H or σ^E. Furthermore, pilin levels were measured after overnight growth on solid media where σ factor expression was induced continuously throughout the growth. Next step would be to perform the analysis of pilin levels in liquid cultures where σ factor upregulation can be variable and controlled over time.

Lastly, σ factors orchestrate a transcriptional response, however in the present study we only examined pilin protein levels and not *pilE* transcript. Therefore regulation of *pilE* transcript in the σ factor mutation backgrounds should be addressed and these experiments are in progress. In addition, it is worth examining the *pilE* TSS in the context of σ mutants as induction of σ^{70} family members may reveal additional promoter sites upstream of *pilE*.

6 Results: Characterisation of σ^H-regulon in *N. meningitidis*

6.1 Introduction

Studying the role of σ factors in *pilE* regulation led me to consider more broadly the transcriptional response of *N. meningitidis* during host adaptation and pathogenicity. Specifically, I decided to study heat stress and the role of σ^H, the putative heat shock response σ factor in *N. meningitidis*.

Adaptation to temperature is thought to play a major role in the meningococcal lifestyle (Lappann *et al.*, 2016; Loh *et al.*, 2013). When *N. meningitidis* colonises the upper respiratory tract, it is exposed to temperatures around 32-34°C which can fluctuate depending on the temperature of the inhaled air (Keck *et al.*, 2000; Virji, 2009) (Figure 6.1). When meningococcus successfully colonises the host and proliferates on the surface of nasopharyngeal epithelia, it can occasionally shift from being a commensal to an invasive pathogen. In these rare cases *N. meningitidis* crosses the epithelial and endothelial barrier, and reaches the bloodstream where it causes systemic disease. During invasion and disease progression, *N. meningitidis* is exposed to different temperatures, ranging from 37°C to >40°C during septic shock. Furthermore, in the nasopharynx during viral co-infection, local inflammation may trigger temperature upshift exposing meningococci to a changing environment (Gentile *et al.*, 1998). Therefore, adaptation to temperature is a key strategy for bacterial survival both as a commensal and as a pathogen during disease.

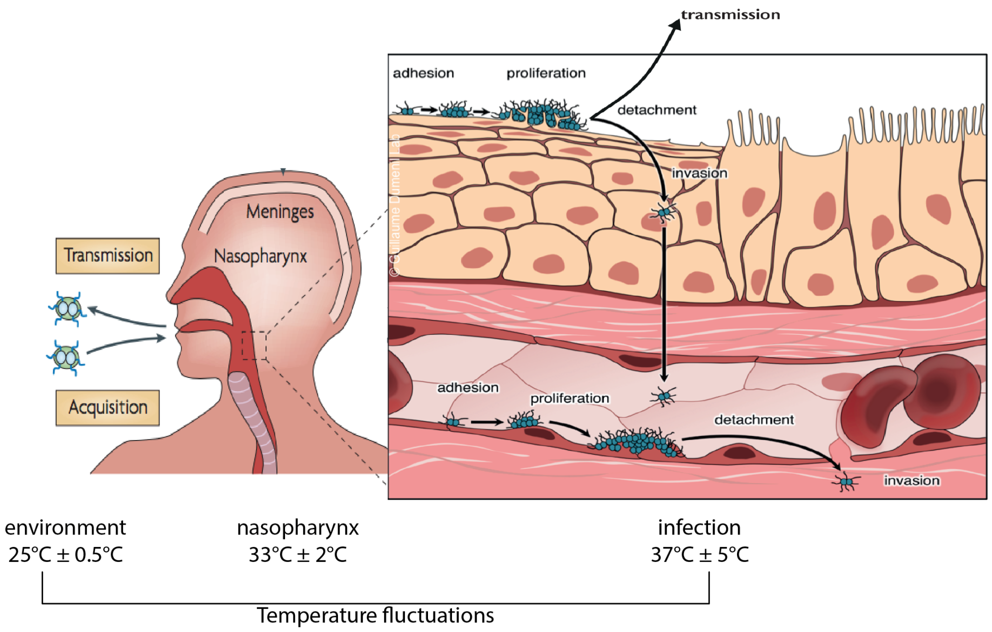


Figure 6.1 *N. meningitidis* exposure to different temperatures within the host

N. meningitidis enters the host via respiratory droplets and resides in the upper respiratory tract where it is exposed to a wider variety of temperatures. Occasionally *N. meningitidis* can cause the invasive disease progressing through the body and entering the brain. Infection of meninges and cerebrospinal fluid may lead to septic shock where bacteria will be exposed to elevated temperatures. Adapted from (Virji, 2009) and Dumenil lab.

The response to temperature in bacteria is complex. It involves multiple temperature sensing strategies that allow bacteria to adapt to sudden temperature changes as well as prolonged heat stress. Temperature sensors include nucleic acids (DNA and RNA), lipids, as well as proteins. RNA thermosensors are temperature sensitive secondary structures present in the 5' end of RNA transcripts which prevent ribosomes from interacting with the mRNA thereby blocking the transcription at lower temperatures. Studies in our lab have shown that some meningococcal virulence genes possess RNA thermosensors. These include *cssA* (polysaccharide biosynthesis gene), *fHbp* (factor H binding protein) and *lst* (enzyme involved in LPS sialylation), which all show increased expression during transition from

32°C to 42°C (Barnwal *et al.*, 2016; Loh *et al.*, 2013). Some organisms possess DNA sensors which function by imposing structural changes on the genome thereby affecting gene transcription. In addition to nucleic acid, bacterial lipid membrane thermosensing mechanisms have been described. For example, in *Bacillus subtilis*, the transmembrane thermosensor DesK can modulate the fluidity of the membrane which in turn affects the cellular response to the environment (Inda *et al.*, 2014; Sengupta *et al.*, 2013). The principal mediators of heat sensing and heat shock response in bacteria are heat shock proteins (HSP). In *E. coli* HSP include virulence factors, proteases, some metabolic regulators and chaperones (Arsene *et al.*, 2000; Guisbert *et al.*, 2004). In *E. coli* the master regulator of heat shock response is σ^H, which is present in low amounts under steady state but increases rapidly during heat shock.

6.1.1 σ^H in *E. coli*

σ^H recognises a subset of heat shock genes that contain a defined -35/-10 consensus sequence: TTGAAA $(N)_{14}$ CCCATAT (Nonaka *et al.*, 2006). The regulation of heat shock genes is controlled by activity and stability of σ^H that in turn responds to temperature change in multiple ways (Figure 6.2). Firstly σ^H is regulated at the level of transcription. There are five (P1, P3-P6) promoters upstream of *rpoH* which are controlled by different σ factors. The P1 promoter can be transcribed by σ^S or housekeeping σ^D depending on the growth phase. P3 is σ^E-dependent linking the regulation of *rpoH* with membrane stress response. P4 and P5 are regulated by σ^D whereas P6 is under the control of σ^N (Janaszak *et al.*, 2009; Nagai *et al.*, 1990). Secondly, *rpoH* mRNA contains a thermosensor which forms three stem loops (Kortmann *et al.*, 2012). Temperature change from 30°C to 42°C destabilises the secondary structure of the mRNA leading to translation initiation. σ^H protein is highly unstable and under steady state it is sequestered by major heat shock chaperones DnaKJE and GroELS (Yura, 1996), which direct σ^H to FtsH protease-dependent degradation, thereby preventing σ^H from interacting with RNAP. Within the first 5 min of elevated temperatures, as misfolded proteins accumulate, the chaperones are titrated away and σ^H becomes free to activate σ^H-dependent genes. This results in upregulation of HSP allowing bacteria to respond adequately to temperature stress. Once the cellular homeostasis is restored, chaperones bind σ^H, decreasing free σ^H levels *via* a negative feedback loop. Recent experiments revealed additional mechanisms of regulation of σ^H stability: rather than being a cytoplasmic σ factor, σ^H is tethered to the inner membrane *via* signal recognition particle (SRP) that in turn is bound by SRP receptor (SR) (Lim *et al.*, 2013; Miyazaki *et al.*, 2016). The interaction between σ^H and SRP is mediated *via* homeostatic control region, which is a short motif within Region 2.1 of σ^H important for σ^H activity (Obrist *et al.*, 2005; Yura *et al.*, 2007). It has been proposed that σ^H tethering to the membrane may allow σ^H to respond not only to accumulation of misfolded proteins in the cytoplasm but also to monitor membrane protein unfolding during heat stress (Roncarati *et al.*, 2017).

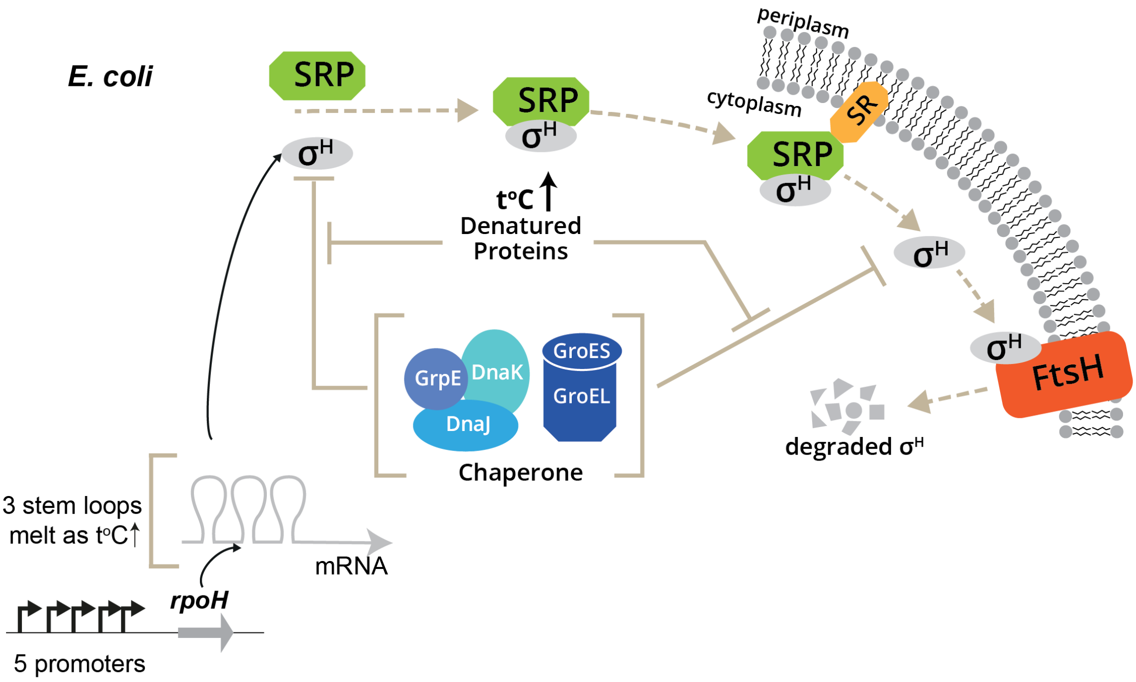


Figure 6.2 σ^H control in *E. coli*

Schematic diagram of σ^H control in *E. coli*. σ^H is regulated at the level of transcription with five promoter described. Post-transcriptionally *rpoH* mRNA contains three stem loops which are melted in response to elevated temperature leading to upregulation of mRNA. Feedback control loops control σ^H concentration during steady state and in response to high temperature. Adapted from (Roncarati & Scarlato, 2017).

There is limited information available on the function and regulation of σ^H in *Neisseria*. Early studies using RNA dot blot hybridization in a closely related pathogen *N. gonorrhoeae* showed that the expression of *rpoH* increased gradually upon exposure to heat over 20 min (Laskos *et al.*, 2004). However subsequent microarray analysis did not identify any upregulation of *rpoH* transcript when *N. gonorrhoeae* was exposed to heat shock for 10 min (Gunesekere *et al.*, 2006) suggesting that RNA thermosensing regulation is absent in *N. gonorrhoeae*. Induction of *N. gonorrhoeae* σ^H leads to upregulation of only a small subset of genes, some of which are involved in protein homeostasis including the chaperone DnaKJE and GroELS systems (Gunesekere *et al.*, 2006). Furthermore, most of these genes showed increased expression when *N. gonorrhoeae* was exposed to 42°C, suggesting a link between σ^H regulon and heat stress response. Despite the attempts to characterise *N. gonorrhoeae* σ^H regulon, σ^H-dependent promoter consensus elements have not been identified in *N. gonorrhoeae* or *N. meningitidis* (Gunesekere *et al.*, 2006; Heidrich *et al.*, 2017) and the role of σ^H in *N. meningitidis* has not been investigated.

The aim of my experiments in this chapter is to establish whether meningococcal σ^H is a heat shock factor and define the σ^H regulon in *N. meningitidis*.

6.2 σ^H role in heat shock response in *N. meningitidis*

The bioinformatic analysis presented in chapter 5 revealed that there is 98% sequence identity between *N. meningitidis* and *E. coli* σ^H suggesting that some functional similarities may be conserved among the two species. Unlike *E. coli*, where σ^H is only essential at >40°C (Yura *et al.*, 1984), *N. meningitidis* σ^H is essential at 37°C, as demonstrated by the failure to construct a *rpoH* null mutant (Capel *et al.*, 2016; Pelicic *et al.*, 2000), and has been proposed to be transcribed from a σ^{70}-dependent promoter (Heidrich *et al.*, 2017). Preliminary bioinformatic analysis of σ^H and its regulatory regions in *N. meningitidis* performed in this study demonstrated that, similar to *E. coli*, *N. meningitidis* σ^H contains the homeostatic control region (78% sequence identity with *E. coli* region) and the homologues of the SRP/SR system (67%/48% sequence identity, respectively) (Figure 6.3), implying that meningococcal σ^H may also be bound to the inner membrane and respond to accumulation of the cytoplasmic and membrane misfolded protein in the same manner as *E. coli* σ^H.

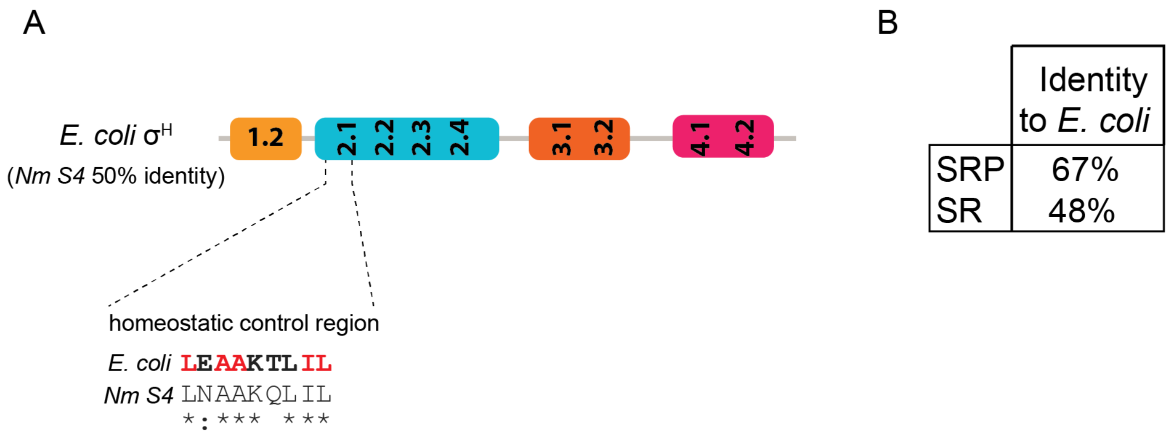


Figure 6.3 σ^H homeostatic control region and SRP/SR homologs in *N. meningitidis*

A. Homeostatic control region alignment between σ^H *E. coli* and *N. meningitidis* S4. Key residues involved in σ^H regulation in *E. coli* are shown in red (Miyazaki *et al.*, 2016). B. Amino acid sequence identity (%) between *E. coli* SRP/SR and putative homologs *N. meningitidis* S4.

Since *E. coli* σ^H is known to be regulated by higher temperatures (Yura, 1996), we first determined whether meningococcal σ^H is induced upon heat shock. *N. meningitidis* S4 $\sigma^{H\text{-}FLAG}$ described in chapter 5 was grown in BHI broth at 37°C until mid-exponential phase (OD$_{600=}$0.4-0.6). At that point, the culture was split into four equal volumes of 15 ml (Figure 6.4A). Two subcultures were subjected to two different heat stress conditions: transient heat shock (42°C for 20 min) and prolonged heat shock (42°C for 50 min) (Figure 6.4A). The remaining two 15 ml subcultures served as controls and were exposed to 37°C for either 20 min (control 1) or 50 min (control 2). RNA and protein samples were prepared from the four subcultures. Two controls were used because bacteria grown for 20 min at either 37°C or 42°C reach a different OD$_{600}$ compared to cultures grown for 50 min at either 37°C or 42°C. Therefore, in addition to temperature, bacterial growth is another variable that could affect the analysis. To account for this, we compared RNA and protein levels of σ^H between samples collected at 37°C after 20 min or 50 min (control 1 vs control 2) and between transient (42°C for 20min) and prolonged heat shock (42°C for 50 min).

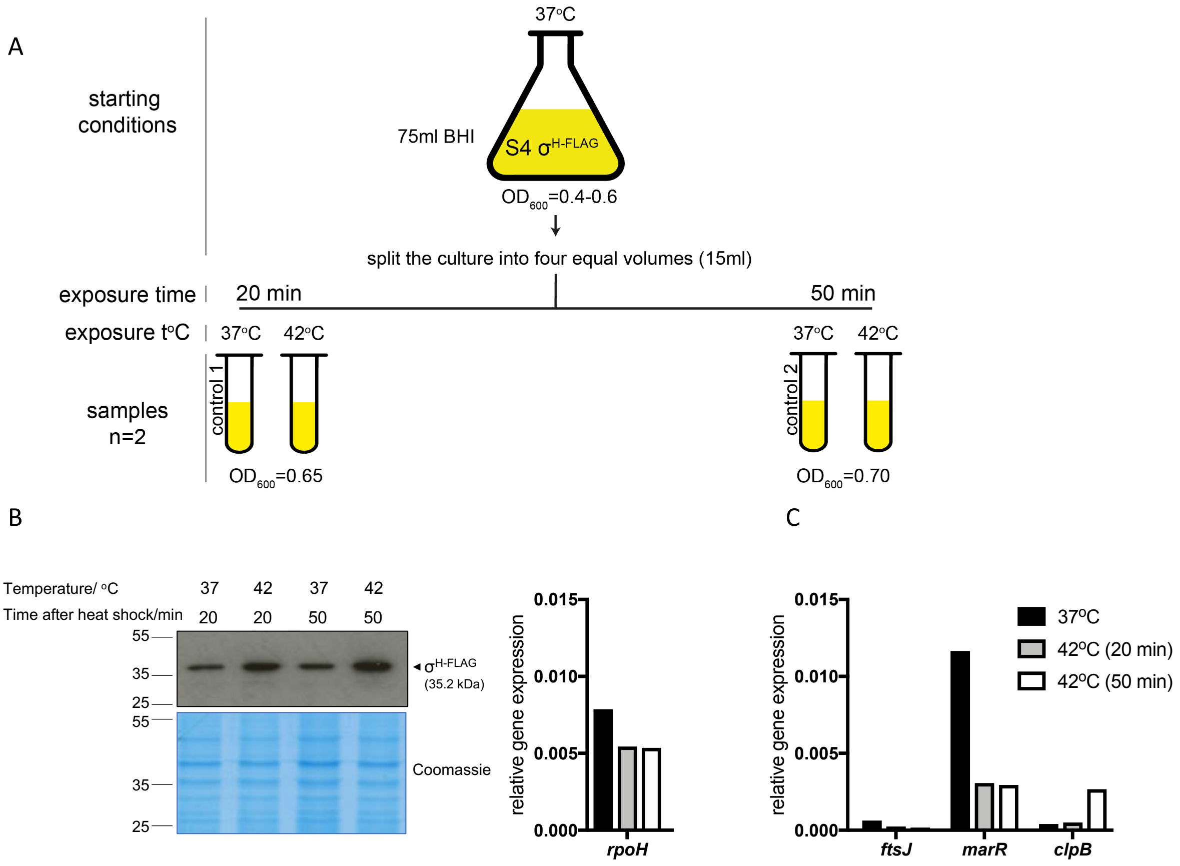


Figure 6.4 Meningococcal σ^H in the heat shock response

A. Schematic workflow for generating samples from *N. meningitidis* S4 $\sigma^{H\text{-}FLAG}$ exposed to transient (42°C for 20 min) and prolonged (42°C for 50 min) heat stress. Bacteria were grown at 37°C until mid-logarithmic phase, and then split into four equal volumes (15 ml) which were incubated at 37°C and 42°C either for 20 min or 50 min. Samples were collected to analyse protein and mRNA. B. Western blot analysis of σ^H expression at 42°C after 20 min or 50 min. 12% acrylamide gels were used to fractionate the cell lysates and protein expression was detected using anti-FLAG antibody. The experiment is a representative of two biological repeats. Relative mRNA expression of *rpoH* was measured by qRT-PCR relative to tmRNA control (n=2). C. The expression of candidate genes *ftsJ*, *marR* and *clpB* was measured using strand-specific qRT-PCR and levels were expressed relative to tmRNA. The experiment is a representative of two biological repeats.

σ^H levels were assessed by Western blotting (Figure 6.4B) which suggested that σ^H is upregulated at 42°C compared to 37°C. Interestingly, the longer exposure to temperature was also associated with higher σ^H levels (Figure 6.4B). σ^H expression levels were comparable between two control group samples (Figure 6.4B, n=2). Next we aimed to determine whether temperature-dependent σ^H regulation can also be observed at the transcriptional level. There was no apparent difference in *rpoH* expression following heat stress compared to expression at 37°C (Figure 6.4B, n=2), suggesting that heat-driven upregulation of σ^H in *N. meningitis* detected at 42°C is mediated post-transcriptionally.

Previous analysis of σ^H in *N. gonorrhoeae* identified 11 genes belonging to the σ^H regulon (Gunesekere *et al.*, 2006). Of these, 8 genes have also been shown to be upregulated upon exposure to 42°C for 10 min (Gunesekere *et al.*, 2006). To determine whether similar overlap between heat shock regulon and σ^H regulon can be detected in *N. meningitidis*, we analysed the expression of several homologues of gonococcal heat shock and σ^H-dependent genes using the RNA prepared as described in Figure 6.4A. Three candidate genes were chosen: a) *clpB* (chaperone) and *marR* (transcriptional regulator) which belong to the gonococcal σ^H regulon and are also induced by heat stress and b) *ftsJ* (cell division protein) which is induced by elevated temperature but is not a member of the gonococcal σ^H regulon (Gunesekere *et al.*, 2006). Expression of these candidate genes was assessed by qRT-PCR using tmRNA as a control. The relative expression of these genes differed from the expression pattern found in *N. gonorrhoeae*: *ftsJ* remained unaffected by either transient or prolonged exposure to heat stress whereas expression of *marR* seemed to be downregulated in response to heat shock (n=2). Only *clpB* expression was upregulated in *N. meningitidis* similar to *N. gonorrhoeae*; however the upregulation was only observed after prolonged (50 min) exposure to 42°C in *N. meningitidis* as opposed to gonococcal *clpB* upregulation, which occurs after 10 min of heat stress (Figure 6.4C). However, these observations are drawn from two biological repeats and in order to establish whether there is any statistically significant difference in relative gene expression additional experiments will be performed with more biological repeats.

Based on our analysis of σ^{H} protein levels during elevated temperature, we can conclude that σ^{H} levels are upregulated in response to increased temperature in *N. meningitidis* and therefore σ^{H} could be implicated in the adaptation to heat stress.

6.3 Differential gene expression analysis in σ^{H} overexpressing strain (RNA-seq)

6.3.1 Designing a system for σ^{H} regulon analysis in *N. meningitidis*

Next, we aimed to identify the σ^{H} regulon in *N. meningitidis* using RNA-seq. Since we were unable to make *N. meningitidis* Δ*rpoH* mutant, the σ^{H} regulon was determined by inducing *rpoH* expression rather than comparing a null mutant with the wild-type. $S4P_{lac}\sigma^{H\text{-}FLAG}$ contains a second copy of *rpoH* at an ectopic locus under the control of an IPTG inducible promoter. The strain was grown in BHI broth at 37°C until mid-logarithmic phase, then IPTG was added for 10 min to induce the expression of the additional copy of *rpoH*. Based on studies in *E. coli* 10 min is optimal time to measure σ^{H} transcriptional response (Nonaka *et al.*, 2006; Yura *et al.*, 1984). An isogenic strain was similarly grown until mid-logarithmic phase with no addition of IPTG and was used as a control (Figure 6.5).

Difference in gene expression between $S4P_{lac}\sigma^{H\text{-}FLAG}$, where *rpoH* is induced with IPTG, and $S4P_{lac}\sigma^{H\_FLAG}$ grown without IPTG, could result from: 1) upregulation of σ^{H} protein upon IPTG induction of *rpoH*, or 2) addition of IPTG inducer for 10 min, or 3) disruption of the ectopic locus by insertion of additional DNA sequence. Therefore, to ensure the present analysis identifies changes in gene expression that result solely from the upregulation of σ^H, S4*ery* strain was used as a control (Figure 6.5). S4$P_{lac}\sigma^{H\text{-}FLAG}$ contains erythromycin selection marker downstream of the *rpoH* gene at the ectopic locus. Therefore, S4*ery* strain was constructed containing only the erythromycin resistance cassette at the same ectopic locus to account for any changes in gene expression that could result from the polar effect of the ectopic locus disruption. S4*ery* culture was grown in duplicates to mid logarithmic phase and IPTG was added to one of the cultures for 10 min (Figure 6.4). Comparing the samples taken from S4ery with or without IPTG would allow us to determine whether differential gene expression detected resulted from the addition of IPTG and/or ectopic locus disruption. By excluding these genes from the differentially expressed genes identified by comparing S4$P_{lac}\sigma^{H\text{-}FLAG}$ samples with or without IPTG, we can reliably determine σ^H-dependent gene expression.

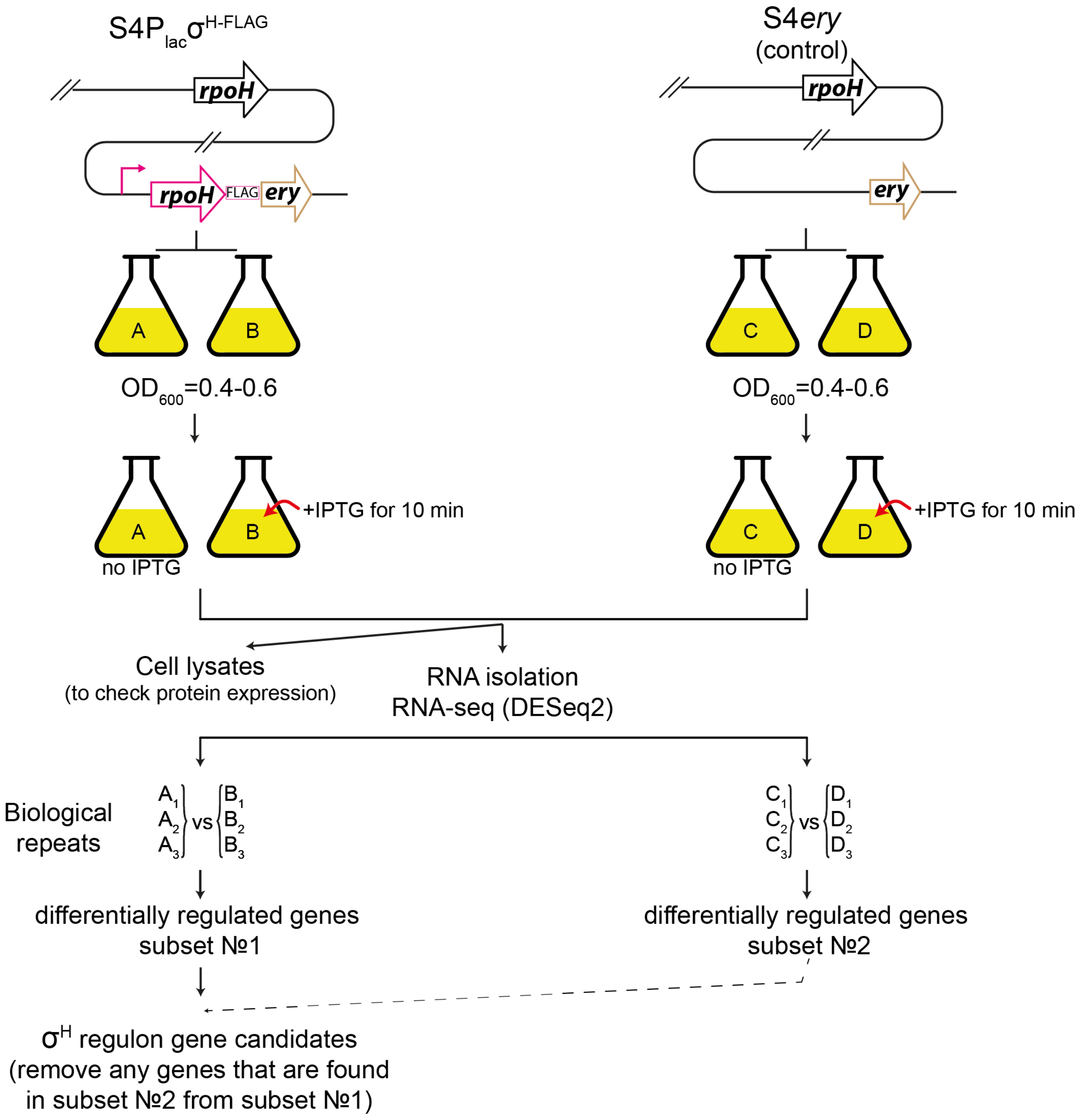


Figure 6.5 Library preparation from *N. meningitidis* S4P$_{lac}$σ$^{H\text{-}FLAG}$ and S4*ery*

Duplicate bacterial cultures grown in BHI until the OD$_{600}$ reached 0.4-0.6 and IPTG was added for 10 min to one flask. Cell lysates were prepare for Western blot analysis of σ^{H} expression and bacterial pellets were collected for RNA extraction. Total RNA from three independent biological repeats was used for library preparation and sequencing and was subjected to differential gene expression analysis using DESeq2 workflow (Love *et al*., 2014). RNA-seq analysis resulted in identification of two subsets of differentially expressed genes: subset №1 and subset №2. Any candidate genes that were present in both subsets were excluded (subset №1- subset №2) therefore allowing the identification of σ^{H}-dependent genes.

Initially the overexpression of σ^H system was validated by examining FLAG-tagged σ^H levels by Western blotting (Figure 6.6A). Tagged σ^H was detected in S4$P_{lac}\sigma^{H\text{-}FLAG}$ treated with IPTG for 10 min which demonstrated that 10 min is sufficient to induce σ^H expression. Using qRT-PCR we also examined *rpoH* mRNA levels in S4$P_{lac}\sigma^{H\text{-}FLAG}$ with or without IPTG. Relative mRNA levels of *rpoH* increase significantly in the IPTG-induced S4$P_{lac}\sigma^{H\text{-}FLAG}$ further validating the system (Figure 6.6B).

Before conducting global RNA-seq analysis, we also examined the expression of *clpB* as we know from the previous result (Figure 6.4C) that *clpB* is upregulated in response to heat stress (n=2) and therefore, similarly to *N. gonorrhoeae*, could also be a member of meningococcal σ^H regulon. *clpB* mRNA levels were comparable between IPTG induced and non-induced S4$P_{lac}\sigma^{H\text{-}FLAG}$ samples suggesting that *clpB*, despite being upregulated in response to elevated temperature, might not be under the control of σ^H. This result suggests that *N. meningitidis* has σ^H-independent mechanisms of responding to heat shock which might be different from *N. gonorrhoeae*.

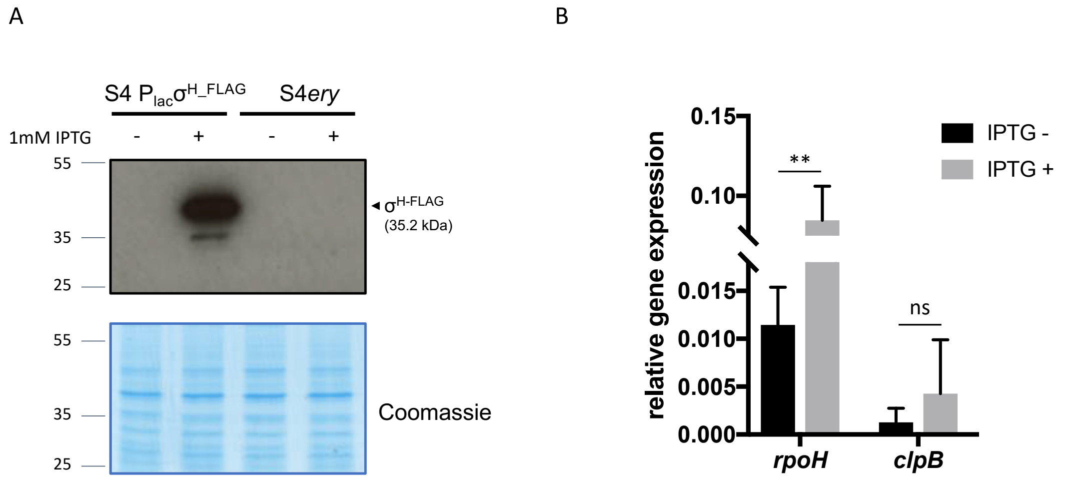


Figure 6.6 σ^H overexpression in *N. meningitidis* S4 $P_{lac}\sigma^{H\text{-}FLAG}$

A. Western blotting of σ^H levels using an anti-FLAG antibody and Coomassie as a loading control. The blot is a representative from three biological repeats. B. RNA isolated from S4 $P_{lac}\sigma^{H\text{-}FLAG}$ grown with or without IPTG was analysed by qRT-PCR for *rpoH* and *clpB* mRNA. Transcript abundance is shown as relative to tmRNA. The experiment is a representative of three repeats, performed three times with three independent biological samples and analysed by Student's t-test where **p value=0.0043, ns is p>0.05.

6.3.2 Differential RNA-seq analysis of σ^H regulon

To identify genes regulated by σ^H, total RNA from bacterial pellets was isolated from samples as described in Figure 6.5 and subjected to RNA-seq analysis (Vertis Biotechnologies). RNA-seq approach for studying σ-dependent gene expression is preferable because it offers a number of advantages: a) it has a higher throughput, as unlike commercial microarray technology it is not restricted to mRNA transcripts, b) it displays high resolution allowing detection of single nucleotide variations as well as identifying transcription start sites (using additional treatment (Sharma *et al.*, 2010); not performed here) and c) unlike hybridisation methods, it does not require detailed knowledge about the reference genome that is used for transcript mapping. Altogether it allows accurate quantification and annotation and has previously been used to define gene expression in *N. meningitidis* (Capel *et al.*, 2016; Heidrich *et al.*, 2017).

Library preparation and sequencing were performed by Vertis Biotechnologie AG (Germany) using Illumina NextSeq 500 system and a 75 bp read length. Sequencing reads were processed by first trimming the adapter sequences and then mapping the reads to a reference genome. Two reference genomes were used for mapping the reads: the *N. meningitidis* 8013 (NC_017501) genome and *N. meningitidis* FAM18 (NC_008767), the latter belongs to the same clonal complex as the S4 isolate used in the study. Transcription of each gene was presented in reads per kilobase per million mapped reads (RPKM) and differentially expressed genes were identified using the DESeq2 workflow (Love *et al.*, 2014). Gene expression levels were presented as $\log_2$ fold change ($\log_2$ FC) and for convenience a fold change (FC) value was calculated using the following formula: $FC=2^{\log_2 FC}$. Statistical analysis was performed to take into account multiple testing and the resulting *p* value was presented as *p* adjusted (*p* adj) (Love *et al.*, 2014).

In order to interpret the RNA-seq data, we arbitrarily considered that following σ^H overexpression, a gene having a $\log_2$ FC > 1 and adjusted *p* value of < 0.05 was positively regulated by *rpoH*, while a gene with $\log_2$ FC < -1 was negatively regulated. In total 172 transcripts were found to be differentially regulated upon σ^H induction with adjusted *p* value < 0.05. Of these, no genes were identified as negatively regulated by σ^H (no genes had $\log_2$ FC < -1) and only two transcripts, *rpoH* itself and NMC_RS08415 (putative type II toxin-antitoxin system Phd/YefM family antitoxin), had $\log_2$ FC value > 1. The biological system designed here contains a native copy of *rpoH* and we predict that it is expressed, based on our observation of the native *rpoH* mRNA levels (Chapter 5, Figure 5.4). Thus there is a possibility that upregulation of *rpoH* from the ectopic locus may result in only subtle induction of putative candidate genes. For this reason we adjusted the threshold values to $\log_2$ FC > 0.1 and adjusted *p* value of < 0.05 for any gene to be considered positively regulated after σ^H induction. Since σ^H is known to be an activator of the heat shock response in in *E. coli* (Nonaka *et al.*, 2006) and σ^H induction in *N. gonorrhoeae* did not result in any down-regulated candidate genes (Gunesekere *et al.*, 2006), we did not readjust the threshold for identification of genes which are negatively regulated by σ^H induction. Thus in this study we focused on identifying genes whose expression increases following induction of σ^H.

Using $\log_2$ FC > 0.1 and adjusted *p* value of < 0.05 as the new threshold, we identified 75 ORFs to be upregulated in response to σ^H induction. We utilised a publicly available platform for microbial gene annotation "NeisseriaScope" within MicroScope (Vallenet *et al.*, 2006) (http://www.genoscope.cns.fr/agc/microscope/home/index.php) to a) determine gene clustering based on the annotated function (eggNOG pipeline (Huerta-Cepas *et al.*, 2016)) and b) to identify essential genes using data from *N. meningitidis* 8013 and FAM18 as reference (Minimal Gene Set genomic tool, MicroScope; (Gil *et al.*, 2004)). Among 75 genes upregulated following σ^H overexpression, 25% were involved in cellular processes (Group 1), 28% in information storage and processing (Group 2), 20% in metabolism (Group 3) and 27% were genes of unknown function (Group 4) (Table 6.1).

Group 1. Group 1 genes link the σ^H-dependent regulon with protein homeostasis, which is similar to the *E. coli* σ^H regulon. Genes encoding chaperone systems (*groES*, *groEL*, *dnaJ*) were induced along with the protease encoding gene, *ftsH*, known to degrade σ^H in *E. coli*. Genes encoding the protein transport system, (*secB* and NMC_RS08785) and genes implicated in signal transduction mechanism, (*mazE* and NMC_RS06080) were also found to be induced. Interestingly, σ^H overexpression leads to upregulation of three components of the Tfp machinery: *pilX*, *pilO* and *pilQ* but not *pilE,* indicating that σ^H may play a role in modulating pilus functions including adhesion. In our study σ^H induction led to upregulation of several genes associated with cell wall, membrane and envelope biogenesis (*bamC*, NMC_RS10645, *porA*). This implies that similarly to *E. coli*, *N. meningitidis* may possess a functional cross-talk between the σ^H regulon and the membrane stress response. In *E. coli*, σ^E is a known membrane stress response regulator and during heat shock σ^E upregulation induces *rpoH* expression *via* the *rpoH* σ^E-dependent P3 promoter, thereby linking membrane stress with heat shock response. Unlike *E. coli*, *N. meningitidis rpoH* is not regulated by σ^E (Du *et al.*, 2005; Huis in 't Veld *et al.*, 2011) and our data shows that *rpoE* expression is independent of heat stress (Chapter 5). Therefore further analysis is needed to determine any possible σ^H-σ^E interplay in *N. meningitidis*.

Table 6.1 ***N. meningitidis*** **genes induced following 10 min induction of σ^H**

	ORF[a]	gene	Proposed function[b]	Class description[c]	Essential gene[d]	HSR in *Nm*[e]	Fold change	$\log_2$FC	*p* value	*p*adj
CELLULAR PROCESSES AND SIGNALING										
GROUP 1	NMC_RS10285	*groEL*	molecular chaperone GroEL	Posttranslational modification, protein turnover, chaperones	Yes	Yes	1.499	0.584	4.44E-06	0.000407
	NMC_RS10290	*groES*	molecular chaperone GroES	Posttranslational modification, protein turnover, chaperones	Yes	Yes	1.35	0.433	0.000162	0.005777
	NMC_RS00255	*dnaJ*	molecular chaperone DnaJ	Posttranslational modification, protein turnover, chaperones	Yes	Yes	1.305	0.384	0.001914	0.024657
	NMC_RS02340	*grxC*	glutaredoxin	Posttranslational modification, protein turnover, chaperones		Yes	1.282	0.359	9.55E-05	0.003967
	NMC_RS00025	*fkbP*	FKBP-type peptidyl-prolyl cis-trans isomerase	Posttranslational modification, protein turnover, chaperones			1.235	0.305	0.000791	0.014277
	NMC_RS04005	*ftsH*	ATP-dependent metallopeptidase FtsH/Yme1/Tma family	Posttranslational modification, protein turnover, chaperone	Yes		1.171	0.228	0.002842	0.032327
	NMC_RS02345	*secB*	protein-export protein SecB	Intracellular trafficking, secretion, and vesicular transport		Yes	1.349	0.432	7.45E-08	1.21E-05
	NMC_RS08785		HlyD family type I secretion periplasmic adaptor subunit	Intracellular trafficking, secretion, and vesicular transport			1.258	0.331	0.003237	0.036066
	NMC_RS02225	*pilQ*	pilus assembly protein PilQ	Intracellular trafficking, secretion, and vesicular transport			1.168	0.224	0.001259	0.018146
	NMC_RS02235	*pilO*	pilus assembly protein PilO	Intracellular trafficking, secretion, and vesicular transport			1.164	0.219	0.001663	0.022463
	NMC_RS04430	*pilX*	pilus assembly protein PilX	Cell motility, intracellular trafficking, secretion, and vesicular transport			1.216	0.282	0.005455	0.04999
	NMC_RS02450	*tpsS2*	putative two-partner silent cassette 2	Intracellular trafficking, secretion, and vesicular transport	Yes		1.241	0.312	0.000226	0.006835
	NMC_RS02440	*tpsS1*	putative two-partner silent cassette 1	Intracellular trafficking, secretion, and vesicular transport	Yes		1.271	0.346	0.000665	0.012509
	NMC_RS04805	*bamC*	outer membrane protein assembly factor BamC	Cell wall/membrane/envelope biogenesis			1.247	0.319	2.54E-05	0.001298
	NMC_RS10645		glycosyl transferase family 1	Cell wall/membrane/envelope biogenesis			1.333	0.414	0.000175	0.00589
	NMC_RS07120	*porA*	porin	Cell wall/membrane/envelope biogenesis			1.142	0.192	0.003557	0.037084
	NMC_RS10805		hypothetical protein	Cell cycle control, cell division, chromosome partitioning			1.301	0.38	0.00058	0.011384
	NMC_RS10680	*mazE*	putative PemI-like protein	Signal transduction mechanisms			1.25	0.322	0.001932	0.024657
	NMC_RS06080		nitrate/nitrite sensor histidine kinase	Signal transduction mechanisms			1.158	0.212	0.003304	0.036333
INFORMATION STORAGE AND PROCESSING										
GROUP 2	NMC_RS03540	*rpoH*	RNA polymerase sigma factor RpoH (Sigma-32)	Transcription	Yes	Yes	3.088	1.627	1.05E-116	1.88E-113
	NMC_RS06655		conserved hypothetical protein	Transcription			1.341	0.423	0.000844	0.014498
	NMC_RS04555		putative helix-turn-helix transcriptional regulator	Transcription			1.231	0.299	0.000494	0.010162
	NMC_RS09970		putative LysR family transcriptional regulator	Transcription			1.283	0.359	0.00082	0.014498
	NMC_RS02690		putative helix-turn-helix transcriptional regulator	Transcription			1.252	0.325	0.003827	0.038858
	NMC_RS06485	*rpsA*	30S ribosomal protein S1	Translation, ribosomal structure and biogenesis			1.211	0.276	0.001092	0.016324
	NMC_RS00740	*rpsC*	30S ribosomal protein S3	Translation, ribosomal structure and biogenesis	Yes		1.127	0.173	0.005209	0.048479
	NMC_RS00700	*rpsJ*	30S ribosomal protein S10	Translation, ribosomal structure and biogenesis	Yes		1.261	0.335	4.47E-05	0.001997
	NMC_RS00750	*rpmC*	50S ribosomal protein L29	Translation, ribosomal structure and biogenesis	Yes		1.181	0.24	0.00069	0.012704
	NMC_RS00820	*rpmJ*	50S ribosomal protein L36	Translation, ribosomal structure and biogenesis	Yes	Yes	1.148	0.199	0.001927	0.024657
	NMC_RS00805	*rplO*	50S ribosomal protein L15	Translation, ribosomal structure and biogenesis	Yes		1.283	0.36	3.53E-06	0.00035
	NMC_RS00600	*rsmD*	16S rRNA (guanine(966)-N(2))-methyltransferase RsmD	Translation, ribosomal structure and biogenesis			1.273	0.349	0.000409	0.009384
	NMC_RS07785	*glnS*	glutamine--tRNA ligase/YqeY domain fusion protein	Translation, ribosomal structure and biogenesis	Yes		1.15	0.202	0.003249	0.036066
	NMC_RS10030	*glyQ*	glycine--tRNA ligase subunit alph	Translation, ribosomal structure and biogenesis			1.168	0.224	0.004686	0.044544
	NMC_RS02335	*cafA*	ribonuclease G	Translation, ribosomal structure and biogenesis			1.218	0.284	0.00041	0.009384
	NMC_RS06660	*ruvX*	holliday junction resolvase RuvX	Replication, recombination and repair	Yes		1.342	0.424	0.001711	0.022629
	NMC_RS00815	*infA*	translation initiation factor IF-1	Replication, recombination and repair	Yes		1.179	0.237	0.000473	0.010162
	NMC_RS06490	*ihfB*	integration host factor subunit beta	Replication, recombination and repair	Yes		1.255	0.327	0.000275	0.007566
	NMC_RS03705	*recN*	DNA repair protein RecN	Replication, recombination and repai			1.2	0.263	0.000896	0.015107
	NMC_RS01110	*gyrB*	DNA gyrase subunit B	Replication, recombination and repai	Yes		1.154	0.207	0.004049	0.040555
	NMC_RS07320	*ssB*	single-stranded DNA-binding protein	Replication, recombination and repai	Yes		1.168	0.224	0.005337	0.049417

a. ORF designations from the annotation of FAM18 (accession number NC_008767.1).

b. Proposed function based on sequence annotation (Vallenet *et al.*, 2006).

c. Gene classes determined using FAM18 annotation or annotation of the homologs found in 8013 in NeisseriaScope (Vallenet *et al.*, 2006).

d. Essential genes identified based on the minimal gene set in NeisseriaScope (Gil *et al.*, 2004; Rusniok *et al.*, 2009) and by comparison to homologs in Z5463 (Capel *et al.*, 2016).

e. Heat shock response (HSR) genes previously identified in *N. meningitidis* exposed to 45°C for 5 min (Guckenberger *et al.*, 2002).

Table 6.1 *N. meningitidis* genes induced following 10 min induction of σ^H

	ORF[a]	gene	Proposed function[b]	Class description[c]	Essential gene[d]	HSR in *Nm*[e]	Fold change	$\log_2$FC	*p* value	*p* adj
METABOLISM										
GROUP 3	NMC_RS01120	*prlC*	oligopeptidase A	Amino acid transport and metabolism	Yes		1.316	0.397	0.00035	0.00877
	NMC_RS08095	*hisJ3*	Histidin binding protein	Amino acid transport and metabolism/Signal transduction mechanisms			1.281	0.357	4.30E-05	0.001968
	NMC_RS10925	*asd*	aspartate-semialdehyde dehydrogenase	Amino acid transport and metabolism			1.176	0.234	0.001114	0.016324
	NMC_RS11390		6-phosphogluconate dehydrogenase	Amino acid transport and metabolism; Carbohydrate transport and metabolism			1.202	0.265	0.001064	0.016324
	NMC_RS09295	*putP*	sodium/proline symporter PutP	Amino acid transport and metabolism			1.221	0.288	0.00417	0.041174
	NMC_RS04930	*sdhD*	succinate dehydrogenase	Energy production and conversion			1.295	0.373	0.000174	0.00589
	NMC_RS10815	*ppc*	phosphoenolpyruvate carboxylase	Energy production and conversion			1.173	0.23	0.002189	0.027358
	NMC_RS02745		NADH:ubiquinone reductase (Na(+)-transporting) subunit C	Energy production and conversion	Yes		1.156	0.21	0.003672	0.03771
	NMC_RS07610	*ackA1*	acetate kinase 1 (acetokinase 1)	Energy production and conversion	Yes		1.16	0.214	0.000799	0.014278
	NMC_RS00035		D-2-hydroxyacid dehydrogenase	Energy production and conversion			1.166	0.222	0.004469	0.042933
	NMC_RS08180	*norC*	nitric oxide reductase	Inorganic ion transport and metabolism	Yes		1.311	0.39	0.000101	0.00412
	NMC_RS08175	*norB*	nitric-oxide reductase large subunit	Inorganic ion transport and metabolism	Yes		1.16	0.214	0.003569	0.037084
	NMC_RS08425	*hemO*	biliverdin-producing heme oxygenase	Inorganic ion transport and metabolism			1.281	0.358	0.000383	0.008998
	NMC_RS08420	*hpuB*	putative ligand-gated channel	Inorganic ion transport and metabolism			1.262	0.335	0.001084	0.016324
	NMC_RS06050	*rpe*	ribulose-phosphate 3-epimerase	Carbohydrate transport and metabolism			1.192	0.253	0.004057	0.040555
OTHER										
GROUP 4	NMC_RS08415		type II toxin-antitoxin system Phd/YefM family antitoxin				1.524	0.608	8.23E-13	4.90E-10
	NMC_RS10835		putative sodium:proton antiporter				1.347	0.429	2.62E-06	0.000275
	NMC_RS00260		putative oligopeptide transporter, OPT famil				1.319	0.4	4.92E-06	0.000419
	NMC_RS12560		hypothetical protein				1.34	0.423	4.09E-05	0.001968
	NMC_RS12495		hypothetical protein				1.319	0.399	0.000376	0.008967
	NMC_RS10830		putative SlyX-like protein				1.236	0.305	0.000238	0.006976
	NMC_RS05400		hypothetical protein			Yes	1.28	0.356	0.000265	0.007389
	NMC_RS06255		putative periplasmic protein				1.267	0.341	0.000679	0.012644
	NMC_RS05260		hypothetical protein				1.229	0.297	0.001105	0.016324
	NMC_RS13260		putative acetyltransferase				1.229	0.297	0.001087	0.016324
	NMC_RS10340		hypothetical protein				1.285	0.362	0.002514	0.030417
	NMC_RS10685		type II toxin-antitoxin system PemK/MazF family toxin				1.242	0.312	0.002568	0.030677
	NMC_RS04700		DUF4760 domain-containing protein				1.285	0.361	0.002615	0.03095
	NMC_RS12230		hypothetical protein				1.256	0.329	0.002677	0.031271
	NMC_RS02255		YihA family ribosome biogenesis GTP-binding protein				1.263	0.337	0.004065	0.040555
	NMC_RS06275		Cys-tRNA(Pro) deacylase				1.213	0.278	0.004882	0.045918
	NMC_RS12175		hypothetical protein				1.23	0.298	0.001491	0.020658
	NMC_RS02030		hypothetical protein				1.174	0.231	0.001745	0.022766
	NMC_RS07160		lactate utilization protein C				1.187	0.248	0.004606	0.044012
	NMC_RS00285		hypothetical protein				1.148	0.199	0.00537	0.049469

a. ORF designations from the annotation of FAM18 (accession number NC_008767.1).
b. Proposed function based on sequence annotation (Vallenet *et al.*, 2006).
c. Gene classes determined using FAM18 annotation or annotation of the homologs found in 8013 in NeisseriaScope (Vallenet *et al.*, 2006).
d. Essential genes identified based on the minimal gene set in NeisseriaScope (Gil *et al.*, 2004; Rusniok *et al.*, 2009) and by comparison to homologs in Z5463 (Capel *et al.*, 2016).
e. Heat shock response (HSR) genes previously identified in *N. meningitidis* exposed to 45°C for 5 min (Guckenberger *et al.*, 2002).

***Group* 2.** Several genes in Group 2 are involved in maintaining genome integrity (*ruvX*, *ihfA*, *ihfB*, *recN*, *gyrB*, *ssB*). These genes are not only necessary for bacterial survival but could confer advantage under stress conditions such as heat shock, when temperature may alter the topology of the chromosome. In *E. coli* higher temperatures promote σ^H-dependent induction of a number of transcription regulators and proteins involved in RNA production and stability (Nonaka *et al.*, 2006). Interestingly, 67% of Group 2 genes were found to be involved in transcription and translation. This is in line with *E. coli* where σ^H-dependent upregulation of genes implicated in transcription and translation has been implicated in bacterial resistance to high temperature (Qi *et al.*, 1996).

***Group* 3.** Metabolic genes that form Group 3 were found to be predominantly associated with amino acid transport (*prlC*, *hisJ3*, *asd*, NMC_RS11390 and *putP*) and energy production and conversion (*sdhD*, *ppc*, NMC_RS02745, *ackA1* and NMC_RS00035). Other candidates which were found to be differentially expressed included inorganic transport and metabolism genes (*norC*, *norB*, *hem*O, *hpuB*), and *rpe* associated with carbohydrate transport and metabolism (Derkaoui *et al.*, 2016; Richardson *et al.*, 1999).

***Group 4*.** RNA-seq analysis identified 20 genes that were upregulated in response to σ^H expression whose function is poorly understood. One of these genes *NMC_RS05400* has previously been shown to be upregulated in response to heat stress in *N. meningitidis*, further demonstrating the overlap between σ^H and heat shock regulation in meningococcus.

We also compared our list of upregulated genes with the recently identified library of meningococcal host colonisation genes (Capel *et al.*, 2016). Out of the 75 genes, ten are known to be implicated in adhesion to host epithelial and endothelial cells, suggesting that σ^H may be involved in regulating bacterial adhesion. Strikingly, among 75 upregulated genes 30% were essential (Capel *et al.*, 2016; Vallenet *et al.*, 2006). This indicates that in *N. meningitidis* σ^H-dependent transcription is necessary for cell survival and viability which is consistent with the evidence that *rpoH* itself is essential in *N. meningitidis*. Taken together it raises an interesting hypothesis that meningococcal σ^H may function as an additional housekeeping σ factor by activating the expression of the essential genes under standard growth as well as during stress conditions.

Summary

Understanding the role of σ factors led us to investigate the σ^H regulon in *N. meningitidis*. We found that meningococcal σH expression is increased upon heat stress which is similar to *E. coli*. However, this increase was only observed at the level of protein.

Further evidence that σ^H could be implicated in adaptation to elevated temperature in *N. meningitidis* comes from our RNA-seq analysis of the σ^H regulon. Following the induction of σ^H, we identified a set of genes that have previously been implicated in the heat shock response in other species. Among these candidates are genes encoding chaperone systems GroEL/ES and DnaJ and FtsH protease which has been shown to degrade *rpoH* in *E. coli*.

Our findings suggest that the meningococcal σ^H regulon consists of 75 genes (~4% of the genome). In addition to the previously described heat shock response genes, σ^H expression led to changes in a number of genes involved in a wide range of cellular functions: metabolism, DNA maintenance, transcription, translation and membrane homeostasis. Such multifactorial response to σ^H expression suggests that the σ^H role might extend beyond the heat shock response in *N. meningitidis*. This hypothesis is further supported by the evidence that among 75 upregulated genes, 22 were essential. Thus σ^H may play a major role in maintaining meningococcal homeostasis during both steady state and continuous temperature fluctuations that bacteria is facing as a human-specific pathogen.

Interestingly, of the 75 genes that were positively induced following σ^H upregulation, ten genes have previously been implicated in meningococcal adhesion to host cells (Capel *et al.*, 2016). Among these ten candidates only *NMC_RS07160* has been shown to be a positive regulator of *N. meningitidis* adhesion to epithelial cells. In our analysis, *pilE* was not detected as differentially expressed gene in response to σ^H induction. Nine other candidates were identified as negative regulators of adhesion to either endothelial cells (*pilO*, *cafA*), epithelial cells (*NMC_RS10830*, *NMC_RS00025*, *NMC_RS10340*) or both cell types (*secB*, *NMC_RS10805*, *pilQ*, *NMC_RS10680*). These results suggest that σ^H may be involved modulating adhesion in response to stress stimuli such as temperature upshift. Indeed, previous studies examined the role of the gonococcal σ^H in pathogenesis showed that σ^H is important for cell invasion but not adhesion (Du *et al.*, 2005). Furthermore, recent analysis of *N. meningitidis* proteome demonstrated that induction of *rpoH* does not lead to increased biofilm formation. Altogether σ^H may function by downregulating adhesion during bacterial exposure to stress stimuli.

Current analysis did not explore whether induction of σ^H leads to any changes in sRNA expression. This is particularly important considering that meningococcal sRNAs are known to form an important post-transcriptional regulatory cascade in *Neisseria* and other species

(McClure *et al.*, 2014; Storz *et al.*, 2011). However, the present study identified 20 genes of unknown function that were positively regulated following σ^H induction. It would be interesting to explore the function of these genes in future.

The immediate steps following this RNA-seq analysis include validation of the putative σ^H-dependent target and characterisation of any common promoter features that could constitute meningococcal σ^H-dependent promoter. This would be particularly useful considering that no σ^H consensus sequences have been identified in *N. meningitidis* based on σ^H sequences in *E. coli* (Heidrich *et al.*, 2017). This knowledge will help us elucidate the regulatory mechanisms that govern σ^H activity and the role of this σ factor in *N. meningitidis*.

7 Discussion

Regulation of class II *pilE* gene in *N. meningitidis* has not been investigated before. The main objective of this project was to explore how *N. meningitidis* class II *pilE* is regulated at the transcriptional level. In this work I characterised regulation of class II *pilE*, which contains several putative promoter sequences including -12/-24, extended -10 and -10/-35, annotated based on the consensus sequences found in *E. coli*. The class I *pilE* also contains -12/-24, extended -10 and -10/-35 promoter sequences, however the position, as well as sequence, differs from those found upstream of class II *pilE*. Class I *pilE* transcription is driven by the -10/-35 promoter (Fyfe *et al.*, 1995a) and is thought to be activated by σ^D. However, there is no experimental evidence showing that it is σ^D rather than another σ^{70} family σ factor that is acting on the promoter. In contrast, commensal *N. elongata pilE* contains only a -12/-24 promoter which is activated by σ^N (Rendon *et al.*, 2013). In these species *pilE* also involves Npa, a σ^N activator, which binds upstream to the -12/-24 sequence, and is required for σ^N-dependent transcription. Npa has recently been shown to interact with Neisseria pilus sensor (Nps) kinase to form a two-component response regulatory system that integrates a yet unknown stimulus *via* a phosphorylation cascade to regulate σ^N-dependent *pilE* transcription (Rendón *et al.*, 2018). This model of *pilE* regulation offers a new insight into *Neisseria* transcriptional regulation.

Using bioinformatic analysis we found that the σ^{70}-dependent -10/-35 sequence, and σ^N-dependent -12/-24 sequence, are highly conserved in the promoter region of the class II *pilE* in *N. meningitidis*. Moreover, these class II *pilE* promoters, including the -12/-24 sequence alone, were active when tested in *E. coli* which contains σ^{70} family and σ^N factors. The conservation and activity of the class II *pilE* -12/-24 region was particularly interesting because this promoter is not well conserved in class I *pilE* strains. Thus we hypothesized that the -12/-24 promoter may provide an alternative mechanism of class II *pilE* regulation. To address this hypothesis, we first showed that all meningococcal isolates harbour an *rpoN* gene. But unlike *N. elongata*, pathogenic *Neisseria* species harbour an *rpoN* gene which encodes a σ^N which lacks the HTH domain mediating -12 sequence recognition, but retains the -24 sequence recognition domain known as the RpoN box. This suggests that promoter interaction could still take place. However, our analysis of class II *pilE* levels in Δ*rpoN* or σ^N overexpressing mutants of *N. meningitidis* revealed that neither the absence nor the overexpression of σ^N affected pilin expression. Furthermore, wild type pilin levels were detected in *N. meningitidis* strain harbouring a deletion of the homolog of the commensal Neisseria σ^N activator-encoding gene (*npa*). This is in contrast to *N. elongata* where the deletion of Npa has been shown to downregulate *pilE* expression (Rendon *et al.*, 2013). Taken together our data suggests that class II *pilE* is not regulated by σ^N. Primer extension analysis supported this observation by demonstrating that class II *pilE* transcription is initiated from the -10/-35 σ^{70}-dependent promoter. We attempted to determine which σ^{70} family member drives *pilE* transcription. Our analysis indicates that σ^D is likely to drive class II *pilE* transcription as changes in the concentration of other σ^{70}-like factors (σ^E and σ^H) did not alter pilin expression. However we do not exclude the possibility that σ^H and σ^E could play a role in pilin regulation under a specific stimulus. Detailed analysis of the interaction of σ^{70}-like factors with *pilE* promoter sequence will benefit our understanding of class II *pilE* transcription initiation.

Our findings indicate that meningococcal σ^N does not function as a canonical σ^N. It is unable to activate a known *E. coli* σ^N-dependent promoter, despite the presence of the RpoN box. Furthermore, we conclude that meningococcal σ^N is unlikely to form any interaction with the consensus σ^N-dependent -12/-24 sequence, because no downregulation of the -12/-24 promoter activity was observed when both *E. coli* and *N. meningitidis* σ^N are expressed in *E. coli*. Whether meningococcal σ^N has acquired an alternative function remains to be determined. Our data shows that meningococcal *rpoN* mRNA is present throughout bacterial growth, but since no protein could be detected, *rpoN* in *N. meningitidis* could serve a regulatory role at the RNA level, or could be a pseudogene.

In parallel we considered other elements that could regulate class II *pilE* transcription. Several transcription factors have previously been implicated in class I *pilE* regulation, including the RNA chaperone Hfq and the transcriptional regulator CrgA (Deghmane *et al.*, 2000; Pannekoek *et al.*, 2009). Using a bioinformatic analysis of meningococcal genomes with class II *pilE*, we identified eight candidate transcription factors including Hfq and CrgA homologs, all containing a HTH DNA binding domain and/or implicated in adhesion (Capel *et al.*, 2016). By examining pilin levels in these transcription factor mutant backgrounds, only the deletion of *hfq* leads to a decreased expression of pilin. Interestingly, recent reports identify Hfq-dependent sRNA in the class I *pilE* locus as a candidate for *pilE* regulation (Heidrich *et al.*, 2017). Thus further work is needed to determine the exact mechanism of Hfq-dependent downregulation of class II pilin.

In addition to transcription factors, earlier studies reported that gonococcal pilin is regulated by several stresses such as high osmolarity, temperature, acid stress and iron depletion

(Larribe *et al.*, 1997). Furthermore, high osmolarity has been shown to downregulate class I *pilE* expression in *N. meningitidis* (Tan *et al.*, 2015). Therefore, we exposed class II *pilE* containing *N. meningitidis* to a panel of stress conditions and examined *pilE* mRNA levels. In line with previous reports in class I *pilE* expressing *N. meningitidis*, high osmolarity lowers *pilE* transcript levels. Interestingly, acid stress (pH 2.5) upregulated class II *pilE* mRNA. It was beyond the scope of the present analysis to determine the mechanisms underlying the response to these stresses. However, future work will focus on understanding the signaling network that allows bacteria to sense the stimuli and trigger differential *pilE* expression. Collectively this knowledge will contribute to our understanding of meningococcal adaptation to niches within the host under steady state and during disease.

An important observation that emerged from our analysis is that in a variety of *N. meningitidis* mutant backgrounds, as well in the presence of extreme stresses, *pilE* expression was never abolished. Considering the importance of Tfp for bacteria-bacteria interactions, DNA competence and adhesion to host cells, we speculate that pathogenic *N. meningitidis* may have adopted a constitutive expression of *pilE* to increase its fitness and preserve virulence capacity. Truncation of *rpoN* and loss of σ^N-dependent transcription from the -12/-24 sequence upstream of class II *pilE* might be an evolutionary way to circumvent negative regulation of class II *pilE*. Our work showing that expression of *N. elongata* σ^N in meningococcus downregulates *pilE* supports this hypothesis. We propose that pathogenic *N. meningitidis* is undergoing a loss of σ^N-dependent function through the loss of activators and DNA binding motifs. At present there are no other genes apart from *pilE* that are known to be activated by σ^N in *N. meningitidis*. Identifying other σ^N-dependent commensal genes will allow us to draw comparisons between commensal gene regulation and regulation of the homologues in pathogenic *Neisseria*. Understanding the distinct regulatory cascades that govern homologous genes may provide additional clues about what differentiates commensal from pathogenic *Neisseria*.

Studying σ^N function in *N. meningitidis* led me to consider other σ factors in *N. meningitidis*. Three σ^{70} family σ factors have been previously identified in *N. meningitidis*: σ^D, σ^H (both essential) and σ^E. The bioinformatic analysis performed in this study identified that *rpoD*, *rpoH* and *rpoE* genes are conserved among *N. meningitidis* isolates and share high sequence identity with genes in commensal species, apart from σ^E which exhibited the lowest homology between *N. meningitidis* and *N. elongata*.

The role of σ^H in *N. meningitidis* has not been described and recent reports to define the primary transcriptome of *N. meningitidis* failed to bioinformatically identify any meningococcal σ^H specific promoter recognition motifs (Heidrich *et al.*, 2017). Considering that adaptation to temperature fluctuations is vital for *N. meningitidis* during both asymptomatic carriage and disease, I focused my subsequent analysis on studying σ^H expression at the elevated temperatures and characterizing the σ^H regulon in *N. meningitidis*.

We identified that upon exposure to high temperature (42°C), σ^H expression was increased in *N. meningitidis*. Interestingly however, unlike in *E. coli*, *N. meningitidis rpoH* mRNA was not induced in elevated temperatures. Several protein homologues which are known to control σ^H levels in *E. coli* have been identified in *N. meningitidis* including GroEL/DnaKJ chaperones, signal recognition particle (SRP) and the FtsH protease. It would be interesting to examine the mechanisms of meningococcal σ^H regulation and compare them with the well-defined regulatory loops described in *E. coli*.

Having determined that meningococcal σ^H is implicated in the heat shock response, we examined whether σ^H induction can impact gene expression. We used RNA-seq to define the σ^H regulon in *N. meningitidis* S4 strain. 172 transcripts were differentially regulated following σ^H induction. In the present work we only considered genes that were positively regulated by σ^H overexpression ($\log_2$ FC>0.1) and out of the 172 transcripts 75 were identified as upregulated. Bioinformatic clustering of these genes based on the annotation in other *N. meningitidis* isolates resulted in identification of four distinct groups associated with 1) cellular processes, 2) information storage and processing, 3) metabolism and 4) uncharacterised genes. Genes upregulated in response to σ^H induction included candidates that have previously been described to be upregulated in response to heat stress in *N. meningitidis*, suggesting that σ^H plays a role in the heat shock response. Interestingly, the presence of differentially regulated genes which are not known to be involved in adaptation to heat stress indicates that σ^H-dependent regulation in *N. meningitidis* might function beyond the canonical heat shock regulation. One of the most striking observations that emerged from this analysis was that 29% of the upregulated genes are essential. Taking into account that σ^H is essential itself, it is possible to predict that σ^H is an important regulator of general meningococcal homeostasis under both steady state and in response to elevated temperature. In future, it would be interesting to explore the extent of the overlap between heat stress and the σ^H regulon in *N. meningitidis*.

The most differentially expressed gene in the RNA-seq analysis presented in this thesis had $\log_2$ FC=1.5 (NMC_RS08415). There are several possibilities why the relative upregulation levels did not display higher $\log_2$ FC values. Firstly, the present system contains the endogenous copy of *rpoH*. Therefore, endogenous σ^H may already "saturate" the promoters: assuming that under steady state enough σ^H is made, then σ^H-dependent promoters are already occupied by σ^H-RNAP complex which leads to gene expression. Any further increase in σ^H may have little impact on those promoters as saturated promoters become relatively insensitive to changes in σ factor presence (Mauri & Klumpp, 2014). Promoter saturation has been proposed as a mechanism to mediate constitutive gene expression thereby protecting some genes from variation of the factor levels in response to external stimuli.

Secondly, it may be that σ^H-dependent regulation is more pronounced when the concentration of σ^H reaches a certain threshold. Even though here we induce σ^H expression from an ectopic locus, we did not quantitatively assess the number of σ^H molecules present in the system. Furthermore, σ^H was induced for 10 min which has been shown to be sufficient for *E. coli* σ^H to activate its regulon. However, the dynamic range of meningococcal σ^H-dependent promoters is not known. Examining gene expression following σ^H induction over time would provide answers to how quickly σ^H is degraded and when σ^H-dependent regulation reaches its peak in *N. meningitidis*. Experiments to test whether the top 10 upregulated candidate genes identified in the RNA-seq analysis change their expression with time following σ^H induction will be performed in future.

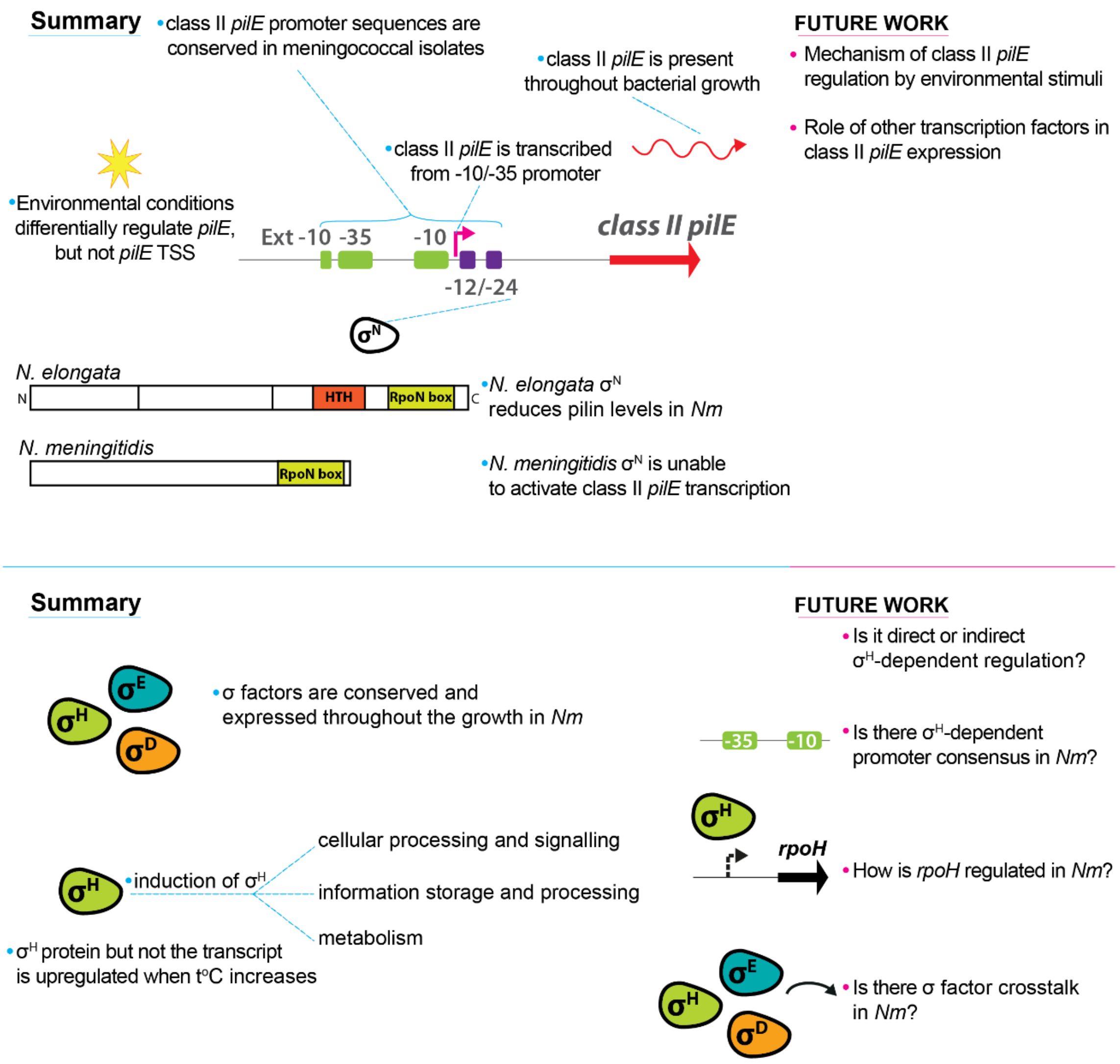
Thirdly, one of the major limitations of these analyses is studying bacteria in a laboratory setting. We conducted our analysis at 37^{o}C in BHI broth, which only partially mimics the physiological state. Therefore, in the physiological settings upregulation of the genes presented in the analysis might be more pronounced. We are currently focusing on validation of the candidate genes and future work will be directed towards characterization of any specific phenotype associated with σ^H induction.

Validation of the candidate genes identified in the RNA-seq is also necessary to determine whether σ^H induction leads to differential expression of the genes *via* direct binding to the promoters upstream of the genes or indirectly *via* σ^H effectors. Future work will involve annotation of the putative promoters upstream of the candidate genes using primer extension. Based on the preliminary analysis of the 50 nt sequences upstream of the start codon of the top 13 upregulated genes, we could not identify a consensus σ^H-dependent promoter sequence homologous to that in *E. coli*. This is consistent with the observation that in *N. meningitidis* 8013 there are only σ^D- or σ^E-dependent consensus promoter elements (Heidrich *et al.*, 2017). We hypothesise that σ^H might not contain a defined promoter binding site in *N. meningitidis*. Instead, it might be able to recognise multiple σ^{70} dependent promoter regions however the successful activation is determined not only by the σ^H interaction with the promoter, but also by the upstream sequence that binds transcriptional regulators. In *E. coli* for example regions upstream of the σ^E-dependent promoters have been shown to modulate the activity of the σ^E-dependent genes (Rhodius & Mutalik, 2010). Therefore in our subsequent experiments we will consider putative promoter regions of the σ^H-induced genes as well as regions beyond the promoters that might be involved in regulating promoter strength.

We did not investigate any of the genes that were downregulated following σ^H expression. 97 transcripts were downregulated with $-1<\log_2 FC<0$. This may suggest that σ^H exert some partial repressive function that has not been previously reported in other species. For instance, σ^S which is a known activator of stationary response in *E. coli* has recently been reported to repress the promoter activity by binding to the promoters and occluding the transcription otherwise mediated by σ^D (Lévi-Meyrueis *et al.*, 2015). This in turn provides additional mechanisms of stress adaptation: repressing genes that are dispensable in a stringent response increases the fitness level of bacteria under stress. It would be interesting to investigate whether meningococcal σ^H uses the same regulatory loops.

Finally, genome-scale studies looking at the binding sites of σ^H in *N. meningitidis* using ChIP-seq analysis would facilitate our understanding of meningococcal σ^H regulon. Combining this data with similar ChIP-seq with two other σ^{70} like factors, σ^D and σ^E, will allow us to picture how the transcriptional space is divided between these three regulons and determine whether any σ factor cross-talk is present in *N. meningitidis*. This global analysis will also provide insight into which σ factors are responsible for regulating virulence determinants and how σ factors govern stress-specific adaptation in *N. meningitidis*.

Figure 7.1 Summary of the results and future work



8 Appendix

8.1 Primers used in the study

8.1.1.1 The following oligonucleotides were used in this study. F is Forward primer, R is Reverse primer.

Oligo nucleotide	Sequence 5'-3'	Description
M1	CACGAATTCTTTTCATTCAGCCACAAAT	F for fragment 1 for *rpoN* deletion in S4 with Kanamycin
M2	CCAGGTACCGATAATGTGGGAGAAATTGT	R for fragment 1 for *rpoN* deletion in S4 Kanamycin
M3	CCAGGTACCAATTGTGTCTCAAAATCTCTGA	F for fragment 2 for *rpoN* deletion in S4 Kanamycin
M4	CCAGGTACCCGATGCATGCCAACAGATAA	R for fragment 2 for *rpoN* deletion in S4 Kanamycin
M5	CCAGGTACCTTGCTGAATAATCTTATAAAGAC	F for fragment 3 for *rpoN* deletion in S4 Kanamycin
M6	CCAGGATCCTTTCGGTCATTTCTGATAAAC	R for fragment 3 for *rpoN* deletion in S4 Kanamycin
M7	GATCCTCTAGAGTCGACCTGCAGGCATGCACTTTTAGACGGCATTTTGGCACTG	F for fragment 1 for *rpoE* deletion in S4
M8	CCCGTTGAATATGGCTCATAGTATCGGGAACAAGGTAAT	R for fragment 1 for *rpoE* deletion in S4
M9	AATTACCTTGTTCCCGATACTATGAGCCATATTCAACGGG	F for fragment 2 for *rpoE* deletion in S4
M10	GTAAATACCCGTGCGGTGTTTTAGAAAAACTCATCGAGCATC	R for fragment 2 for *rpoE* deletion in S4
M11	GCTCGATGAGTTTTTCTAAAACACCGCACGGGTATTTAC	F for fragment 3 for *rpoE* deletion in S4
M12	CAGGAAACAGCTATGACCATGATTACGCCATGCTGCTTTTGAAGCGTG	R for fragment 3 for *rpoE* deletion in S4
M13	CAATTGAATCCGAGCTGAACCCTGTTTTG	F for 152 bp *pilE* upstream region
M14	GCATTGAATCCAAATACCCATACCAGACA	F for 199 bp *pilE* upstream region
M15	GCATTGAATCCCCGCGTTGATATAAAAAA	F for 322 bp *pilE* upstream region
M16	CACGGATCCTTGGATGACTCCTGTTTGAA	R for *pilE* promoter *lacZ* fusion construction in pRS415
M17	CAGTCTAGAAGGAGAGTACGATTCTGAACATGACCCTGCTGGGC	F for *rpoN*$^{E.\ coli}$ cloning into pBAD33
M18	CAGAAGCTTTTATTCTGCGGTTTT	R for *rpoN*$^{E.\ coli}$ cloning into pBAD33

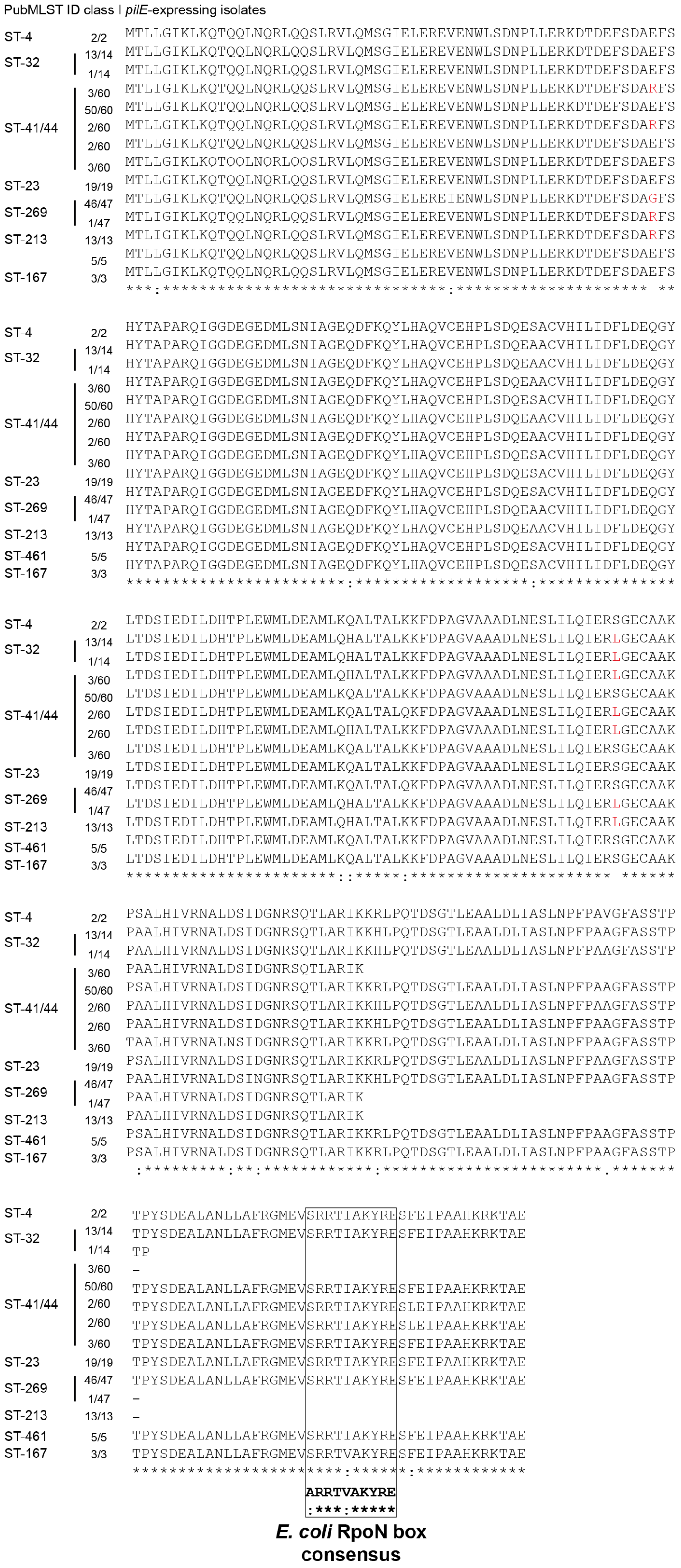
M19	CAGTCTAGAAGGAGAGTACGATTCTGAACAT	F for *rpoN*^{Nm} cloning into pBAD33
M20	CACGCATGCTCAAACGAGTTGTTTACGCTG	R for *rpoN*^{Nm} cloning into pBAD33
M21	GGATCCTGCATAGTCCGGGACGTCATAGGGATAGCCCGCATAGTCAGGAACATCGTATGGGTAAACGAGTTGTTTACGCTGGTT	R for *rpoN*$^{E.coli}$-HA
M22	TATCCCTATGACGTCCCGGACTATGCAGGATCCTATCCATATGACGTTCCAGATTACGCTTAAAAGCTTGGCTGTTTTGGC	F for *rpoN*$^{E.coli}$-HA and *rpoN*^{Nm}-HA
M23	ATAGGATCCTGCATAGTCCGGGACGTCATAGGGATAGCCCGCATAGTCAGGAACATCGTATGGGTATTCTGCGGTTTTACGTTTATGG	R for *rpoN*^{Nm}-HA
M24	AGCTCAACCAGCGGCTGCAACA	F for fragment 1 for *rpoN*-His construction
M25	TTTATAAGATTATTCAGCAATTAGTGGTGGTGGTGGTGGT	R for fragment 1 for *rpoN*-His construction
M26	ACCACCACCACCACCACTAATTGCTGAATAATCTTATAAA	F for fragment 2 for *rpoN*-His construction
M27	CAGAGATTTTGAGACACAATTATTTCTGATAAACCCCCG	R for fragment 2 for *rpoN*-His construction
M28	CGGGGGTTTATCAGAAATAATTGTGTCTCAAAATCTCTG	F fragment 3 for *rpoN*-His construction
M29	GCTTTTGAGTTTCGGTCCGATGCATGCCAACAGATAA	R fragment 3 for *rpoN*-His construction
M30	TTATCTGTTGGCATGCATCGGACCGAAACTCAAAAGC	F fragment 4 for *rpoN*-His construction
M31	GGGCGGAATCGGTCTGGATTCG	R fragment 4 for *rpoN*-His construction
M32	GATCCTCTAGAGTCGACCTGCAGGCATGCACCCAAAGAGGCACAAGACA	F for fragment 1 for *rpoH*-FLAG (endogenous) construction
M33	TCGTTAAATTTTCACTTGTCGTCGTCGTCCTTGTAGTCGATGTCGTGGTCCTTGTAGTCACCGTCGTGGTCCTTGTAGTCAACCGCTTCGGCTTCTTC	R for fragment 1 for *rpoH*-FLAG (endogenous) construction
M34	AAGGACGACGACGACAAGTGAAAATTTAACGATCACTCATCATGT	F for fragment 2 for *rpoH*-FLAG (endogenous) construction
M35	CGACTGGAAAGCGGGCA	R for fragment 2 for *rpoH*-FLAG (endogenous) construction
M36	ACCTCACTGCCCGCTTTCCAGTCGAATATACGCAATAAAATACCCGACAAATTG	F for fragment 3 for *rpoH*-FLAG (endogenous) construction
M37	CAGGAAACAGCTATGACCATGATTACGCCAATATTTCCGCTGCTCATGT	R for fragment 3 *rpoH*-FLAG (endogenous) construction
M38	CCTTGTAGTCACCGTCGTGGTCCTTGTAGTCTTATTCTGCGGTTTTGCGTTT	R for *rpoN*^{Nm} (no FLAG) cloning into pNMC2
M39	TTACGAATTCCCGGATTAATTAAAAGCTTGGAGTAATTTTATGACC	F for *rpoN*^{Nm} (no FLAG) cloning into pNMC2
M40	CCCAAGCTTGGAGTAATTTTATGACCTTACTCGGAATAAAGCT	F for *rpoN*^{Nm}-FLAG and *rpoN*^{Nm} (no FLAG) and cloning into pNMC2

M41	TTATTTTGCTATGAGGGATCCGCTAGCACTAG	R for *rpoN*^{Nm}-FLAG cloning into pNMC2
M42	TTACGAATTCCCGGATTAATTAAAAGCTTGGAGTAATTTTATGACATTGCTCGGATTAAAACT	F for *rpoN*^{Nel} and *rpoN*^{Nel}-FLAG cloning into pNMC2
M43	CTTGTAGTCACCGTCGTGGTCCTTGTAGTCGATCCTGCGTTGGTGTGC	R for *rpoN*^{Nel}-FLAG cloning into pNMC2
M44	CTTGTAGTCACCGTCGTGGTCCTTGTAGTCCTAGATCCTGCGTTGGTGTGC	R for *rpoN*^{Nel} (no FLAG) cloning into pNMC2
M45	ACCATGATTACGAATTCCCGGATTAATTAAAGGAGTAATTTTATGAATAACGCTTTCGC	F for *rpoH*^{Nm}-FLAG and *rpoH*^{Nm} (no FLAG)cloning into pNMC2
M46	TCGTTAAATTTTCACTTGTCGTCGTCGTCCTTGTAGTCGATGTCGTGGTCCTTGTAGTCACCGTCGTGGTCCTTGTAGTCAACCGCTTCGGCTTCTTC	R for *rpoH*^{Nm}-FLAG fragment
M47	GGATCCTCTAGAGTCGACCTGCAGGCATGCATCACTTGTCGTCGTCGTC	R for *rpoH*^{Nm}-FLAG cloning into pNMC2
M48	CCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCATCAAACCGCTTCGGCTTC	R for *rpoH*^{Nm} (no FLAG) cloning into pNMC2
M49	CCATGATTACGAATTCCCGGATTAATTAAAGGAGTAATTTTATGCCGCTACCCGACCT	F for *rpoE*^{Nm}-FLAG and *rpoE*^{Nm} (no FLAG) cloning into pNMC2
M50	GATCCTCTAGAGTCGACCTGCAGGCATGCATTACTTCGGGTTTTCTTGGTTGA	R for *rpoE*^{Nm} (no FLAG) cloning into pNMC2
M51	TCACTTGTCGTCGTCGTCCTTGTAGTCGATGTCGTGGTCCTTGTAGTCACCGTCGTGGTCCTTGTAGTCCTTCGGGTTTTCTTGGTTGAAC	R for *rpoE*^{Nm}-FLAG cloning into pNMC2
M52	CGCGGAATTCTCATGTTTGACAGCTTATAAGCGCGAAGACTTGAACCAC	F for fragment 1 pNMC2 construction
M53	CTAGTGCTAGCGGATCCCTCATAGCAAAATAAAATGCCGT	R for fragment 1 pNMC2 construction
M54	TTATTTTGCTATGAGGGATCCGCTAGCACTAG	F for fragment 2 pNMC2 construction
M55	AACAAAATTTAACGATCACTCATCATGTTC	R for fragment 2 pNMC2 construction
M56	GAACATGATGAGTGATCGTTAAATTTTGTTAATCCACTATAAAAATGCC	F for fragment 3 pNMC2 construction
M57	GAGATTTCGATTCCACCGCCGCCTTATGCAGTCAGCACCAATAC	R for fragment 3 pNMC2 construction
M58	AAGGCGGCGGTGGAATCG	F for fragment 4 pNMC2 construction
M59	ATAAGCTGTCAAACATGAGAATTCCGC	R for fragment 4 pNMC2 construction
ML77	ATTAACCAATTCTGAGCCCTTCCCGGGGA	R for fragment 2 for *rpoN* deletion in S4 with erythromycin
ML78	TCCCCGGGAAGGGCTCAGAATTGGTTAAT	F for fragment 3 for *rpoN* deletion in S4 with erythromycin
ML79	GCGGGAATTCGATATCCCACCAACCCACT	F for fragment 1 for P*pilE-LacZ*

ML80	TGAATCCGTAATCATGGTCATTTGGATGACTCCTG	R for fragment 1 for P*pilE-LacZ*
ML81	CAGGAGTCATCCAAATGACCATGATTACGGATTCA	F for fragment 2 for P*pilE-LacZ*
ML82	TGTCCTTATTTGGTGTTATTTTTGACACCA	R for fragment 2 for P*pilE-LacZ*
ML83	TGGTGTCAAAAATAACACCAAATAAGGACA	F for fragment 3 for P*pilE-LacZ*
ML84	CGAATTCACTAGTGATTCACGATAGGCGAG	R for fragment 3 for P*pilE-LacZ*

8.2 Alignment of deduced amino acid sequences of σ^N in *N. meningitidis*

For full dataset description see Chapter 3. Amino acids are coloured based on their degree of conservation (red: non-conserved residues,). RpoN box is in brackets and RpoN box consensus sequence is shown in bold.



```
PubMLST ID class I pilE-expressing isolates

ST-4      2/2    MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-32     13/14  MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
          1/14   MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
          3/60   MTLIGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
          50/60  MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-41/44  2/60   MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
          2/60   MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
          3/60   MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-23     19/19  MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-269    46/47  MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREIENWLSDNPLLERKDTDEFSDAGFS
          1/47   MTLIGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
ST-213    13/13  MTLIGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
          5/5    MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-167    3/3    MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
                 ***:*******************************:********************* **

ST-4      2/2    HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-32     13/14  HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
          1/14   HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
          3/60   HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
          50/60  HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-41/44  2/60   HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
          2/60   HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
          3/60   HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-23     19/19  HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-269    46/47  HYTAPARQIGGDEGEDMLSNIAGEEDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
          1/47   HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
ST-213    13/13  HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
ST-461    5/5    HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-167    3/3    HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
                 ************************:*******************:***************

ST-4      2/2    LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-32     13/14  LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLNESLILQIERLGECAAK
          1/14   LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLNESLILQIERLGECAAK
          3/60   LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLNESLILQIERLGECAAK
          50/60  LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-41/44  2/60   LTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVAAADLNESLILQIERLGECAAK
          2/60   LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLNESLILQIERLGECAAK
          3/60   LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-23     19/19  LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-269    46/47  LTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVAAADLNESLILQIERSGECAAK
          1/47   LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLNESLILQIERLGECAAK
ST-213    13/13  LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLNESLILQIERLGECAAK
ST-461    5/5    LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-167    3/3    LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
                 ***********************::*****:********************** ******

ST-4      2/2    PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAVGFASSTP
ST-32     13/14  PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
          1/14   PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
          3/60   PAALHIVRNALDSIDGNRSQTLARIK
          50/60  PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-41/44  2/60   PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
          2/60   PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
          3/60   TAALHIVRNALNSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-23     19/19  PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-269    46/47  PAALHIVRNALDSINGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
          1/47   PAALHIVRNALDSIDGNRSQTLARIK
ST-213    13/13  PAALHIVRNALDSIDGNRSQTLARIK
ST-461    5/5    PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-167    3/3    PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
                  :*********:**:************:************************.*******

ST-4      2/2    TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-32     13/14  TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
          1/14   TP
          3/60   -
          50/60  TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-41/44  2/60   TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
          2/60   TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
          3/60   TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-23     19/19  TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-269    46/47  TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
          1/47   -
ST-213    13/13  -
ST-461    5/5    TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-167    3/3    TPYSDEALANLLAFRGMEVSRRTVAKYRESFEIPAAHKRKTAE
                 ***********************:******:************
                                    ARRTVAKYRE
                                    :***:*****
```

PubMLST ID class I *pilE*-expressing isolates

```
ST-364   1/1 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREIENWLSDNPLLERKDTDEFSDAGFS
ST-198   1/1 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-1157  1/1 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
ST-1136  1/1 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-18    1/1 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-226   1/1 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
ST-865   1/1 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-37    1/1 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
ST-103   4/4 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-282   2/2 MTLLGYKLKQTQQLDRRLQQSLRVLQMSGIELEREVEDWLSDNPLLERKDTDEFSDAGFS
ST-22    3/3 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-35    1/5 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
         4/5 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDPDEFSDAGFS
ST-60    8/8 MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
             ***** ********::*******************:*:************ ****** **

ST-364   1/1 HYTAPARQIGGDEGEDMLSNIAGEEDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-198   1/1 HYTAPACQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
ST-1157  1/1 HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
ST-1136  1/1 HYTAPACQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
ST-18    1/1 HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-226   1/1 HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
ST-865   1/1 HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-37    1/1 HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
ST-103   4/4 HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-282   2/2 HYTAPARQIGGDEGEDMLSNIAGEEDFKQYLHAQVCEHPLPDQEAACVHILIDFLDEQGY
ST-22    3/3 HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-35    1/5 HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
         4/5 HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
ST-60    8/8 HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
             ****** *****************:*************** ***:***************

ST-364   1/1 LTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVAAADLNESLILQIERSGECAAK
ST-198   1/1 LTDSIEDILDHTPLEWMLDEAMLQHELTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-1157  1/1 LTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVAAADLNESLILQIERLGECAAK
ST-1136  1/1 LTDSIEDILDHTPLEWMLDEAMLQHELTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-18    1/1 LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-226   1/1 LTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVAAADLNESLILQIERLGECAAK
ST-865   1/1 LTDSIEDILDHTPLEWMLDEAMLQHELTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-37    1/1 LTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVAAADLNESLILQIERLGECAAK
ST-103   4/4 LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-282   2/2 LTDSIEDILDHTPLEWMLDEAMLQHELTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-22    3/3 LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-35    1/5 LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLTESLILQIERLGECAAK
         4/5 LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLNESLILQIERLGECAAK
ST-60    8/8 LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
             ***********************:: ****:************.********* ******

ST-364   1/1 PAALHIVRNALDSIDGNRSQTLARIKKHLP
ST-198   1/1 PAALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-1157  1/1 PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-1136  1/1 PAALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-18    1/1 PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-226   1/1 PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-865   1/1 PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-37    1/1 PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-103   4/4 PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-282   2/2 TAALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-22    3/3 PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-35    1/5 PSALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
         4/5 PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-60    8/8 PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
              :*************************:********************************

ST-364   1/1 -
ST-198   1/1 TPYSDEALANLLAFRGMEVSRRTVAKYRESFEIPAAHKRKTAE
ST-1157  1/1 TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
ST-1136  1/1 TPYSDEALANLLAFRGMEVSRRTVAKYRESFEIPAAHKRKTAE
ST-18    1/1 TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-226   1/1 TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
ST-865   1/1 TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-37    1/1 TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
ST-103   4/4 TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-282   2/2 TPYSDEALANLLAFRGMEVSRRTVAKYRESFEIPAAHKRKTAE
ST-22    3/3 TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-35    1/5 TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
         4/5 TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-60    8/8 TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
             ***********************:******:************
                                ARRTVAKYRE
                                :***:*****
```

***E. coli* RpoN box consensus**

PubMLST ID class I *pilE*-expressing isolates

```
422           MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
417           MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREIENWLSDNPLLERKDTDEFSDAGFS
19965         MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDPDEFSDAGFS
20060         MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
20227         MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
20321         MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
20354         MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
20367         MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
              ***********************************:************** ****** **

422           HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
417           HYTAPVRQIGGDEGEDMLSNIAGEEDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
19965         HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
20060         HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
20227         HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEASCVHILIDFLDEQGY
20321         HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
20354         HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
20367         HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEYPLSDQEAACVHILIDFLDEQGY
              *****.******************:************:******::**************

422           LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
417           LTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVAAADLNESLILQIERSGECAAK
19965         LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLNESLILQIERLGECAAK
20060         LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLTESLILQIERLGECAAK
20227         LTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVAAADLNESLILQIERSGECAAK
20321         LTDSIEDILDHTPLEWMLDEAMLQHELTALKKFDPAGVAAADLNESLILQIERSGECAAK
20354         LTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVAAADLNESLILQIERLGECAAK
20367         LTDSIEDILDHTPLEWMSDEAMLQHALTALKKFDPAGVAAADLNESLILQIERLGECAAK
              ***************** *****:: ****:************.********* ******

422           PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
417           PAALHIVRNALDSINGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
19965         PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
20060         PSALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
20227         PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
20321         PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
20354         PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
20367         PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
              *:************:************:********************************

422           TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
417           TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
19965         TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
20060         TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
20227         TPYSDEALANLLVFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
20321         TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
20354         TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
20367         TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
              ************.******************:***********
                                 ARRTVAKYRE
                                 :***:*****
```

***E. coli* RpoN box consensus**

PubMLST ID class II *pilE*-expressing isolates

```
ST-11 complex/ET-37 complex 9/33   MTLLGYKLKQTQQLNQRLQQSLRVLQMSGIELEREVEDWLSDNPLLERKDTDEFSDAEFS
                            7/33   MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
                            17/33  MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
ST-4 complex/subgroup IV    1/1    MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-1 complex/subgroup I/II  2/14   MTLLGIKLKQTQ----RLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDARFS
                            10/14  MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
                            1/14   MTLLGYKLKQTQQLDRRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
                            1/14   MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-8 complex/Cluster A4     4/8    MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
                            4/8    MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-5 complex/subgroup III   12/12  MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
ST-174 complex              9/9    MTLLGIKLKQTQQLNQRLQQSLRVLQMSGIELEREVENWLSDNPLLERKDTDEFSDAEFS
                                   ***** ******     *********************:*****************.**

ST-11 complex/ET-37 complex 9/33   HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
                            7/33   HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
                            17/33  HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
ST-4 complex/subgroup IV    1/1    HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-1 complex/subgroup I/II  2/14   HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
                            10/14  HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQEAACVHILIDFLDEQGY
                            1/14   HYTAPARQIGGDEGEDMLSNIAGEEDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
                            1/14   HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-8 complex/Cluster A4     4/8    HYTAPARQIGGDEGEDMLSNIAGEEDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
                            4/8    HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-5 complex/subgroup III   12/12  HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
ST-174 complex              9/9    HYTAPARQIGGDEGEDMLSNIAGEQDFKQYLHAQVCEHPLSDQESACVHILIDFLDEQGY
                                   ************************:*******************:***************

ST-11 complex/ET-37 complex 9/33   LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERLGECAAK
                            7/33   LTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVAAADLNESLILQIERSGECAAK
                            17/33  LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-4 complex/subgroup IV    1/1    LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-1 complex/subgroup I/II  2/14   LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLNESLILQIERLGECAAK
                            10/14  LTDSIEDILDHTPLEWMLDEAMLQHALTALKKFDPAGVAAADLNESLILQIERLGECAAK
                            1/14   LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
                            1/14   LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-8 complex/Cluster A4     4/8    LTDSIEDILDHTPLEWMLDEAMLKQALTALQKFDPAGVAAADLNESLILQIERSGECAAK
                            4/8    LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-5 complex/subgroup III   12/12  LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
ST-174 complex              9/9    LTDSIEDILDHTPLEWMLDEAMLKQALTALKKFDPAGVAAADLNESLILQIERSGECAAK
                                   ***********************::*****:********************** ******

ST-11 complex/ET-37 complex 9/33   PSALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
                            7/33   PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
                            17/33  PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-4 complex/subgroup IV    1/1    PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAVGFASSTP
ST-1 complex/subgroup I/II  2/14   PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
                            10/14  PAALHIVRNALDSIDGNRSQTLARIKKHLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
                            1/14   PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
                            1/14   PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-8 complex/Cluster A4     4/8    PAALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
                            4/8    TTALHIVRNALNSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
ST-5 complex/subgroup III   12/12  PSALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAVGFASSTP
ST-174 complex              9/9    PAALHIVRNALDSIDGNRSQTLARIKKRLPQTDSGTLEAALDLIASLNPFPAAGFASSTP
                                    :*********:***************:************************.*******

ST-11 complex/ET-37 complex 9/33   TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
                            7/33   TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
                            17/33  TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
ST-4 complex/subgroup IV    1/1    TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-1 complex/subgroup I/II  2/14   TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
                            10/14  TPYSDEALANLLAFRGMEVSRRTIAKYRESLEIPAAHKRKTAE
                            1/14   TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
                            1/14   TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-8 complex/Cluster A4     4/8    TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
                            4/8    TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-5 complex/subgroup III   12/12  TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
ST-174 complex              9/9    TPYSDEALANLLAFRGMEVSRRTIAKYRESFEIPAAHKRKTAE
                                   ******************************:************

                                                        ARRTVAKYRE
                                                        :***:*****
```

***E. coli* RpoN box consensus**

9 Bibliography

Aho, E. L., Botten, J. W., Hall, R. J., Larson, M. K., & Ness, J. K. (1997). Characterization of a class II pilin expression locus from *Neisseria meningitidis*: evidence for increased diversity among pilin genes in pathogenic *Neisseria* species. *Infect Immun, 65*(7), 2613-2620.

Aho, E. L., Keating, A. M., & McGillivray, S. M. (2000). A comparative analysis of pilin genes from pathogenic and nonpathogenic *Neisseria* species. *Microb Pathog, 28*(2), 81-88. doi:10.1006/mpat.1999.0325

Anna, S., J., S. H., Sascha, D., E., U. L., Heiko, L., & Thorsten, M. (2009). The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) σ factor protein family. *Molecular Microbiology, 74*(3), 557-581. doi:doi:10.1111/j.1365-2958.2009.06870.x

Archibald, F. S., & DeVoe, I. W. (1978). Iron in *Neisseria meningitidis*: minimum requirements, effects of limitation, and characteristics of uptake. *J Bacteriol, 136*(1), 35-48.

Arsene, F., Tomoyasu, T., & Bukau, B. (2000). The heat shock response of *Escherichia coli*. *Int J Food Microbiol, 55*(1-3), 3-9.

Arsène, F., Tomoyasu, T., Mogk, A., Schirra, C., Schulze-Specking, A., & Bukau, B. (1999). Role of Region C in Regulation of the Heat Shock Gene-Specific Sigma Factor of *Escherichia coli* . *Journal of Bacteriology, 181*(11), 3552.

Barne, K. A., Bown, J. A., Busby, S. J., & Minchin, S. D. (1997). Region 2.5 of the *Escherichia coli* RNA polymerase sigma70 subunit is responsible for the recognition of the 'extended-10' motif at promoters. *The EMBO Journal, 16*(13), 4034-4040. doi:10.1093/emboj/16.13.4034

Barnwal, R. P., Loh, E., Godin, K. S., Yip, J., Lavender, H., Tang, C. M., & Varani, G. (2016). Structure and mechanism of a molecular rheostat, an RNA thermometer that modulates immune evasion by Neisseria meningitidis. *Nucleic Acids Research, 44*(19), 9426-9437. doi:10.1093/nar/gkw584

Barrios, H., Valderrama, B., & Morett, E. (1999). Compilation and analysis of σ54-dependent promoter sequences. *Nucleic Acids Research, 27*(22), 4305-4313. doi:10.1093/nar/27.22.4305

Bennett, J. S., Griffiths, D. T., McCarthy, N. D., Sleeman, K. L., Jolley, K. A., Crook, D. W., & Maiden, M. C. (2005). Genetic diversity and carriage dynamics of *Neisseria lactamica* in infants. *Infect Immun, 73*(4), 2424-2432. doi:10.1128/iai.73.4.2424-2432.2005

Bervoets, I., Van Brempt, M., Van Nerom, K., Van Hove, B., Maertens, J., De Mey, M., & Charlier, D. (2018). A sigma factor toolbox for orthogonal gene expression in

Escherichia coli. *Nucleic Acids Research, 46*(4), 2133-2144. doi:10.1093/nar/gky010

Blatter, E. E., Ross, W., Tang, H., Gourse, R. L., & Ebright, R. H. (1994). Domain organization of RNA polymerase alpha subunit: C-terminal 85 amino acids constitute a domain capable of dimerization and DNA binding. *Cell, 78*(5), 889-896.

Bordes, P., Wigneshweraraj, S. R., Schumacher, J., Zhang, X., Chaney, M., & Buck, M. (2003). The ATP hydrolyzing transcription activator phage shock protein F of *Escherichia coli*: identifying a surface that binds sigma 54. *Proc Natl Acad Sci U S A, 100*(5), 2278-2283. doi:10.1073/pnas.0537525100

Borg, J., Christie, D., Coen, P. G., Booy, R., & Viner, R. M. (2009). Outcomes of meningococcal disease in adolescence: prospective, matched-cohort study. *Pediatrics, 123*(3), e502-509. doi:10.1542/peds.2008-0581

Boyle-Vavra, S., So, M., & Seifert, H. S. (1993). Transcriptional control of gonococcal pilE expression: involvement of an alternate sigma factor. *Gene, 137*(2), 233-236.

Brandtzaeg, P., Kierulf, P., Gaustad, P., Skulberg, A., Bruun, J. N., Halvorsen, S., & Sorensen, E. (1989). Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. *J Infect Dis, 159*(2), 195-204.

Brandtzaeg, P., Mollnes, T. E., & Kierulf, P. (1989). Complement activation and endotoxin levels in systemic meningococcal disease. *J Infect Dis, 160*(1), 58-65.

Brissette, J. L., Weiner, L., Ripmaster, T. L., & Model, P. (1991). Characterization and sequence of the *Escherichia coli* stress-induced psp operon. *Journal of molecular biology, 220*(1), 35-48. doi:https://doi.org/10.1016/0022-2836(91)90379-K

Brown, D. R., Helaine, S., Carbonnelle, E., & Pelicic, V. (2010). Systematic Functional Analysis Reveals That a Set of Seven Genes Is Involved in Fine-Tuning of the Multiple Functions Mediated by Type IV Pili in *Neisseria meningitidis*. *Infection and Immunity, 78*(7), 3053-3063. doi:10.1128/iai.00099-10

Browning, D. F., & Busby, S. J. W. (2016). Local and global regulation of transcription initiation in bacteria. *Nature Reviews Microbiology, 14*, 638. doi:10.1038/nrmicro.2016.103

Buck, M. (1986). Deletion analysis of the Klebsiella pneumoniae nitrogenase promoter: importance of spacing between conserved sequences around positions -12 and -24 for activation by the nifA and ntrC (glnG) products. *Journal of Bacteriology, 166*(2), 545.

Buck, M., & Cannon, W. (1992). Specific binding of the transcription factor sigma-54 to promoter DNA. *Nature, 358*, 422. doi:10.1038/358422a0

Buck, M., Gallegos, M.-T., Studholme, D. J., Guo, Y., & Gralla, J. D. (2000). The Bacterial Enhancer-Dependent ς54(ςN) Transcription Factor. *Journal of BActeriology, 182*(15), 4129-4136. doi:10.1128/jb.182.15.4129-4136.2000

Buck, M., Khan, H., & Dixon, R. (1985). Site-directed mutagenesis of the *Klebsiella pneumoniae* nifL and nifH promoters and in vivo analysis of promoter activity. *Nucleic Acids Research, 13*(21), 7621-7638. doi:10.1093/nar/13.21.7621

Buckee, C. O., Jolley, K. A., Recker, M., Penman, B., Kriz, P., Gupta, S., & Maiden, M. C. J. (2008). Role of selection in the emergence of lineages and the evolution of virulence in *Neisseria meningitidis*. *Proceedings of the National Academy of Sciences, 105*(39), 15082.

Bush, M., & Dixon, R. (2012). The Role of Bacterial Enhancer Binding Proteins as Specialized Activators of σ54-Dependent Transcription. *Microbiology and Molecular Biology Reviews, 76*(3), 497-529.

Cahoon, L. A., & Seifert, H. S. (2009). An Alternative DNA Structure Is Necessary for Pilin Antigenic Variation in *Neisseria gonorrhoeae*. *Science, 325*(5941), 764.

Cahoon, L. A., & Seifert, H. S. (2011). Focusing homologous recombination: pilin antigenic variation in the pathogenic *Neisseria*. *Mol Microbiol, 81*(5), 1136-1143. doi:10.1111/j.1365-2958.2011.07773.x

Cahoon, L. A., & Seifert, H. S. (2013). Transcription of a cis-acting, Noncoding, Small RNA Is Required for Pilin Antigenic Variation in *Neisseria gonorrhoeae*. *PLoS Pathog, 9*(1), e1003074. doi:10.1371/journal.ppat.1003074

Campagne, S., Marsh, M. E., Capitani, G., Vorholt, J. A., & Allain, F. H. T. (2014). Structural basis for −10 promoter element melting by environmentally induced sigma factors. *Nature Structural &Amp; Molecular Biology, 21*, 269. doi:10.1038/nsmb.2777
https://www.nature.com/articles/nsmb.2777#supplementary-information

Campbell, E. A., Greenwell, R., Anthony, J. R., Wang, S., Lim, L., Das, K., . . . Darst, S. A. (2007). A Conserved Structural Module Regulates Transcriptional Responses to Diverse Stress Signals in Bacteria. *Molecular Cell, 27*(5), 793-805. doi:10.1016/j.molcel.2007.07.009

Campbell, H., Borrow, R., Salisbury, D., & Miller, E. (2009). Meningococcal C conjugate vaccine: the experience in England and Wales. *Vaccine, 27 Suppl 2*, B20-29. doi:10.1016/j.vaccine.2009.04.067

Cannon, W., Austin, S., Moore, M., & Buck, M. (1995). Identification of close contacts between the sigma N (sigma 54) protein and promoter DNA in closed promoter complexes. *Nucleic Acids Res, 23*(3), 351-356.

Capel, E., Zomer, A. L., Nussbaumer, T., Bole, C., Izac, B., Frapy, E., . . . Coureuil, M. (2016). Comprehensive Identification of Meningococcal Genes and Small Noncoding RNAs Required for Host Cell Colonization. *mBio, 7*(4). doi:10.1128/mBio.01173-16

Carrick, C. S., Fyfe, J. A., & Davies, J. K. (1997). The normally silent sigma54 promoters upstream of the pilE genes of both *Neisseria gonorrhoeae* and *Neisseria meningitidis* are functional when transferred to *Pseudomonas aeruginosa*. *Gene, 198*(1-2), 89-97.

Caugant, D. A. (2008). Genetics and evolution of Neisseria meningitidis: importance for the epidemiology of meningococcal disease. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases, 8*(5), 558-565. doi:10.1016/j.meegid.2008.04.002

Caugant, D. A., & Maiden, M. C. J. (2009). Meningococcal carriage and disease—Population biology and evolution. *Vaccine, 27*(4), B64-B70. doi:10.1016/j.vaccine.2009.04.061

Cehovin, A., Simpson, P. J., McDowell, M. A., Brown, D. R., Noschese, R., Pallett, M., . . . Pelicic, V. (2013). Specific DNA recognition mediated by a type IV pilin. *Proc Natl Acad Sci U S A, 110*(8), 3065-3070. doi:10.1073/pnas.1218832110

Cehovin, A., Winterbotham, M., Lucidarme, J., Borrow, R., Tang, C. M., Exley, R. M., & Pelicic, V. (2010). Sequence conservation of pilus subunits in Neisseria meningitidis. *Vaccine, 28*(30), 4817-4826. doi:10.1016/j.vaccine.2010.04.065

Chamot-Rooke, J., Mikaty, G., Malosse, C., Soyer, M., Dumont, A., Gault, J., . . . Dumenil, G. (2011). Posttranslational modification of pili upon cell contact triggers N. meningitidis dissemination. *Science, 331*(6018), 778-782. doi:10.1126/science.1200729

Chang, Q., Tzeng, Y.-L., & Stephens, D. S. (2012). Meningococcal disease: changes in epidemiology and prevention. *Clinical Epidemiology, 4*, 237-245. doi:10.2147/CLEP.S28410

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., & Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Research, 31*(13), 3497-3500. doi:10.1093/nar/gkg500

Cho, B.-K., Kim, D., Knight, E. M., Zengler, K., & Palsson, B. O. (2014). Genome-scale reconstruction of the sigma factor network in *Escherichia coli*: topology and functional states. *BMC Biology, 12*(1), 4. doi:10.1186/1741-7007-12-4

Christensen, H., May, M., Bowen, L., Hickman, M., & Trotter, C. L. (2010). Meningococcal carriage by age: a systematic review and meta-analysis. *Lancet Infect Dis, 10*(12), 853-861. doi:10.1016/s1473-3099(10)70251-6

Christiansen, J. K., Larsen, M. H., Ingmer, H., Søgaard-Andersen, L., & Kallipolitis, B. H. (2004). The RNA-Binding Protein Hfq of Listeria monocytogenes: Role in Stress Tolerance and Virulence. *Journal of Bacteriology, 186*(11), 3355-3362. doi:10.1128/JB.186.11.3355-3362.2004

Collins, R. F., Davidsen, L., Derrick, J. P., Ford, R. C., & Tønjum, T. (2001). Analysis of the PilQ Secretin from Neisseria meningitidis by

Transmission Electron Microscopy Reveals a Dodecameric Quaternary Structure. *Journal of Bacteriology, 183*(13), 3825.

Contreras, A., & Drummond, M. (1988). The effect on the function of the transcriptional activator NtrC from Klebsiella pneumoniae of mutations in the DNA-recognition helix. *Nucleic Acids Res, 16*(9), 4025-4039.

Coureuil, M., Lecuyer, H., Scott, M. G., Boularan, C., Enslen, H., Soyer, M., . . . Marullo, S. (2010). Meningococcus Hijacks a beta2-adrenoceptor/beta-Arrestin pathway to cross brain microvasculature endothelium. *Cell, 143*(7), 1149-1160. doi:10.1016/j.cell.2010.11.035

Coureuil, M., Mikaty, G., Miller, F., Lecuyer, H., Bernard, C., Bourdoulous, S., . . . Nassif, X. (2009). Meningococcal type IV pili recruit the polarity complex to cross the brain endothelium. *Science, 325*(5936), 83-87. doi:10.1126/science.1173196

Danby, C. S., Cosentino, L. A., Rabe, L. K., Priest, C. L., Damare, K. C., Macio, I. S., . . . Hillier, S. L. (2016). Patterns of Extragenital Chlamydia and Gonorrhea in Women and Men Who Have Sex With Men Reporting a History of Receptive Anal Intercourse. *Sexually Transmitted Diseases, 43*(2), 105-109. doi:10.1097/olq.0000000000000384

Darst, S. A., Opalka, N., Chacon, P., Polyakov, A., Richter, C., Zhang, G., & Wriggers, W. (2002). Conformational flexibility of bacterial RNA polymerase. *Proc Natl Acad Sci U S A, 99*(7), 4296-4301. doi:10.1073/pnas.052054099

Deghmane, A. E., Giorgini, D., Larribe, M., Alonso, J. M., & Taha, M. K. (2002). Down-regulation of pili and capsule of Neisseria meningitidis upon contact with epithelial cells is mediated by CrgA regulatory protein. *Mol Microbiol, 43*(6), 1555-1564.

Deghmane, A. E., Petit, S., Topilko, A., Pereira, Y., Giorgini, D., Larribe, M., & Taha, M.-K. (2000). Intimate adhesion of Neisseria meningitidis to human epithelial cells is under the control of the crgA gene, a novel LysR-type transcriptional regulator. *The EMBO Journal, 19*(5), 1068-1078. doi:10.1093/emboj/19.5.1068

deHaseth, P. L., Zupancic, M. L., & Record, M. T. (1998). RNA Polymerase-Promoter Interactions: the Comings and Goings of RNA Polymerase. *Journal of Bacteriology, 180*(12), 3019-3025.

Derkaoui, M., Antunes, A., Nait Abdallah, J., Poncet, S., Maze, A., Ma Pham, Q. M., . . . Deutscher, J. (2016). Transport and Catabolism of Carbohydrates by Neisseria meningitidis. *J Mol Microbiol Biotechnol, 26*(5), 320-332. doi:10.1159/000447093

Diallo, K., Trotter, C., Timbine, Y., Tamboura, B., Sow, S. O., Issaka, B., . . . Maiden, M. C. J. (2016). Pharyngeal carriage of Neisseria species in the African meningitis belt. *Journal of Infection, 72*(6), 667-677. doi:10.1016/j.jinf.2016.03.010

Dodd, I. B., & Egan, J. B. (1990). Improved detection of helix-turn-helix DNA-binding motifs in protein sequences. *Nucleic Acids Research, 18*(17), 5019-5026.

Doucleff, M., Pelton, J. G., Lee, P. S., Nixon, B. T., & Wemmer, D. E. (2007). Structural Basis of DNA Recognition by the Alternative Sigma-Factor, σ54. *Journal of molecular biology, 369*(4), 1070-1078. doi:10.1016/j.jmb.2007.04.019

Du, Y., Lenz, J., & Arvidson, C. G. (2005). Global gene expression and the role of sigma factors in *Neisseria gonorrhoeae* in interactions with epithelial cells. *Infection and Immunity, 73*(8), 4834-4845. doi:10.1128/iai.73.8.4834-4845.2005

Durfee, T., Hansen, A.-M., Zhi, H., Blattner, F. R., & Jin, D. J. (2008). Transcription Profiling of the Stringent Response in *Escherichia coli*. *Journal of Bacteriology, 190*(3), 1084.

Eriksson, J., Eriksson, O. S., Maudsdotter, L., Palm, O., Engman, J., Sarkissian, T., . . . Jonsson, A.-B. (2015). Characterization of motility and piliation in pathogenic *Neisseria*. *BMC Microbiology, 15*, 92. doi:10.1186/s12866-015-0424-6

Evans, C. M., Pratt, C. B., Matheson, M., Vaughan, T. E., Findlow, J., Borrow, R., . . . Read, R. C. (2011). Nasopharyngeal colonization by Neisseria lactamica and induction of protective immunity against *Neisseria meningitidis*. *Clin Infect Dis, 52*(1), 70-77. doi:10.1093/cid/ciq065

Exley, R. M., Shaw, J., Mowe, E., Sun, Y.-h., West, N. P., Williamson, M., . . . Tang, C. M. (2005). Available carbon source influences the resistance of *Neisseria meningitidis* against complement. *The Journal of Experimental Medicine, 201*(10), 1637-1645. doi:10.1084/jem.20041548

Fantappiè, L., Metruccio, M. M. E., Seib, K. L., Oriente, F., Cartocci, E., Ferlicca, F., . . . Delany, I. (2009). The RNA Chaperone Hfq Is Involved in Stress Response and Virulence in *Neisseria meningitidis* and Is a Pleiotropic Regulator of Protein Expression. *Infection and Immunity, 77*(5), 1842-1853. doi:10.1128/iai.01216-08

Feklistov, A., Bae, B., Hauver, J., Lass-Napiorkowska, A., Kalesse, M., Glaus, F., . . . Darst, S. A. (2017). RNA polymerase motions during promoter melting. *Science, 356*(6340), 863.

Feklistov, A., & Darst, S. A. (2011). Structural basis for promoter-10 element recognition by the bacterial RNA polymerase sigma subunit. *Cell, 147*(6), 1257-1269. doi:10.1016/j.cell.2011.10.041

Feklístov, A., Sharon, B. D., Darst, S. A., & Gross, C. A. (2014). Bacterial Sigma Factors: A Historical, Structural, and Genomic Perspective. *Annual Review of Microbiology, 68*(1), 357-376. doi:10.1146/annurev-micro-092412-155737

Finne, J., Bitter-Suermann, D., Goridis, C., & Finne, U. (1987). An IgG monoclonal antibody to group B meningococci cross-reacts with developmentally regulated polysialic acid units of glycoproteins in neural and extraneural tissues. *J Immunol, 138*(12), 4402-4407.

Fransen, F., Hamstra, H. J., Boog, C. J., van Putten, J. P., van den Dobbelsteen, G. P. J. M., & van der Ley, P. (2010). The Structure of *Neisseria meningitidis*; Lipid A

Determines Outcome in Experimental Meningococcal Disease. *Infect Immun, 78*(7), 3177.

Frosch, M., Weisgerber, C., & Meyer, T. F. (1989). Molecular characterization and expression in *Escherichia coli* of the gene complex encoding the polysaccharide capsule of *Neisseria meningitidis* group B. *Proc Natl Acad Sci U S A, 86*(5), 1669-1673.

Fujita, M., Tanaka, K., Takahashi, H., & Amemura, A. (1994). Transcription of the principal sigma-factor genes, rpoD and rpoS, in *Pseudomonas aeruginosa* is controlled according to the growth phase. *Mol Microbiol, 13*(6), 1071-1077.

Fyfe, J. A., Carrick, C. S., & Davies, J. K. (1995). The *pilE* gene of *Neisseria gonorrhoeae* MS11 is transcribed from a sigma 70 promoter during growth in vitro. *J Bacteriol, 177*(13), 3781-3787.

Fyfe, J. A. M., Strugnell, R. A., & Davies, J. K. (1993). Control of gonococcal pilin-encoding gene expression in *Escherichia coli*. *Gene, 123*(1), 45-50. doi:https://doi.org/10.1016/0378-1119(93)90537-D

Gentile, D., Doyle, W., Whiteside, T., Fireman, P., Hayden, F. G., & Skoner, D. (1998). Increased interleukin-6 levels in nasal lavage samples following experimental influenza A virus infection. *Clin Diagn Lab Immunol, 5*(5), 604-608.

Geoffroy, M.-C., Floquet, S., Métais, A., Nassif, X., & Pelicic, V. (2003). Large-Scale Analysis of the Meningococcus Genome by Gene Disruption: Resistance to Complement-Mediated Lysis. *Genome Research, 13*(3), 391-398. doi:10.1101/gr.664303

Ghosh, P., Ishihama, A., & Chatterji, D. (2001). *Escherichia coli* RNA polymerase subunit omega and its N-terminal domain bind full-length beta' to facilitate incorporation into the alpha2beta subassembly. *Eur J Biochem, 268*(17), 4621-4627.

Gil, R., Silva, F. J., Pereto, J., & Moya, A. (2004). Determination of the core of a minimal bacterial gene set. *Microbiology and Molecular Biology Reviews, 68*(3), 518-537, table of contents. doi:10.1128/mmbr.68.3.518-537.2004

Giuliani, M. M., Adu-Bobie, J., Comanducci, M., Arico, B., Savino, S., Santini, L., . . . Pizza, M. (2006). A universal vaccine for serogroup B meningococcus. *Proc Natl Acad Sci U S A, 103*(29), 10834-10839. doi:10.1073/pnas.0603940103

Glyde, R., Ye, F., Darbari, V. C., Zhang, N., Buck, M., & Zhang, X. (2017). Structures of RNA Polymerase Closed and Intermediate Complexes Reveal Mechanisms of DNA Opening and Transcription Initiation. *Molecular Cell, 67*(1), 106-116.e104. doi:https://doi.org/10.1016/j.molcel.2017.05.010

Glyde, R., Ye, F., Jovanovic, M., Kotta-Loizou, I., Buck, M., & Zhang, X. (2018). Structures of Bacterial RNA Polymerase Complexes Reveal the Mechanism of DNA Loading and Transcription Initiation. *Molecular Cell, 70*(6), 1111-1120.e1113. doi:10.1016/j.molcel.2018.05.021

Gonzalez, V., Olvera, L., Soberon, X., & Morett, E. (1998). In vivo studies on the positive control function of NifA: a conserved hydrophobic amino acid patch at the central domain involved in transcriptional activation. *Mol Microbiol, 28*(1), 55-67.

Gopal, V., & Chatterji, D. (1997). Mutations in the 1.1 subdomain of *Escherichia coli* sigma factor sigma70 and disruption of its overall structure. *Eur J Biochem, 244*(2), 613-618.

Gries, T. J., Kontur, W. S., Capp, M. W., Saecker, R. M., & Record, M. T., Jr. (2010). One-step DNA melting in the RNA polymerase cleft opens the initiation bubble to form an unstable open complex. *Proc Natl Acad Sci U S A, 107*(23), 10418-10423. doi:10.1073/pnas.1000967107

Griffith, K. L., & Wolf, R. E., Jr. (2002). Measuring beta-galactosidase activity in bacteria: cell growth, permeabilization, and enzyme assays in 96-well arrays. *Biochemical and Biophysical Research Communications, 290*(1), 397-402. doi:10.1006/bbrc.2001.6152

Gruber, T. M., & Gross, C. A. (2003). Multiple Sigma Subunits and the Partitioning of Bacterial Transcription Space. *Annual Review of Microbiology, 57*(1), 441-466. doi:10.1146/annurev.micro.57.030502.090913

Guisbert, E., Herman, C., Lu, C. Z., & Gross, C. A. (2004). A chaperone network controls the heat shock response in *E. coli*. *Genes Dev, 18*(22), 2812-2821. doi:10.1101/gad.1219204

Gunesekere, I. C., Kahler, C. M., Powell, D. R., Snyder, L. A., Saunders, N. J., Rood, J. I., & Davies, J. K. (2006). Comparison of the RpoH-dependent regulon and general stress response in *Neisseria gonorrhoeae*. *Journal of Bacteriology, 188*(13), 4769-4776. doi:10.1128/jb.01807-05

Gunesekere, I. C., Kahler, C. M., Ryan, C. S., Snyder, L. A. S., Saunders, N. J., Rood, J. I., & Davies, J. K. (2006a). Ecf, an Alternative Sigma Factor from *Neisseria gonorrhoeae*, Controls Expression of msrAB, Which Encodes Methionine Sulfoxide Reductase. *Journal of Bacteriology, 188*(10), 3463-3469. doi:10.1128/jb.188.10.3463-3469.2006

Haas, R., & Meyer, T. F. (1986). The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: evidence for gene conversion. *Cell, 44*(1), 107-115.

Hammerschmidt, S., Muller, A., Sillmann, H., Muhlenhoff, M., Borrow, R., Fox, A., . . . Frosch, M. (1996). Capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (siaD): correlation with bacterial invasion and the outbreak of meningococcal disease. *Mol Microbiol, 20*(6), 1211-1220.

Harrison, L. H., Trotter, C. L., & Ramsay, M. E. (2009). Global epidemiology of meningococcal disease. *Vaccine, 27 Suppl 2*, B51-63. doi:10.1016/j.vaccine.2009.04.063

Harrison, O. B., Claus, H., Jiang, Y., Bennett, J. S., Bratcher, H. B., Jolley, K. A., . . . Maiden, M. C. J. (2013). Description and Nomenclature of *Neisseria meningitidis* Capsule Locus. *Emerging Infectious Diseases, 19*(4), 566-573. doi:10.3201/eid1904.111799

Harrison, O. B., Cole, K., Peters, J., Cresswell, F., Dean, G., Eyre, D. W., . . . Maiden, M. C. J. (2017). Genomic analysis of urogenital and rectal *Neisseria meningitidis* isolates reveals encapsulated hyperinvasive meningococci and coincident multidrug-resistant gonococci. *Sexually Transmitted Infections, 93*(6), 445.

Hawley, D. K., & McClure, W. R. (1983). Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res, 11*(8), 2237-2255.

Haydon, D. J., & Guest, J. R. (1991). A new family of bacterial regulatory proteins. *FEMS Microbiol Lett, 63*(2-3), 291-295.

Heckman, K. L., & Pease, L. R. (2007). Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat Protoc, 2*(4), 924-932. doi:10.1038/nprot.2007.132

Heidrich, N., Bauriedl, S., Barquist, L., Li, L., Schoen, C., & Vogel, J. (2017). The primary transcriptome of *Neisseria meningitidis* and its interaction with the RNA chaperone Hfq. *Nucleic Acids Research*. doi:10.1093/nar/gkx168

Helaine, S., Carbonnelle, E., Prouvensier, L., Beretti, J. L., Nassif, X., & Pelicic, V. (2005). PilX, a pilus-associated protein essential for bacterial aggregation, is a key to pilus-facilitated attachment of *Neisseria meningitidis* to human cells. *Mol Microbiol, 55*(1), 65-77. doi:10.1111/j.1365-2958.2004.04372.x

Helm, R. A., & Seifert, H. S. (2010). Frequency and rate of pilin antigenic variation of *Neisseria meningitidis*. *J Bacteriol, 192*(14), 3822-3823. doi:10.1128/jb.00280-10

Helmann, J. D., & Chamberlin, M. J. (1988). Structure and function of bacterial sigma factors. *Annu Rev Biochem, 57*, 839-872. doi:10.1146/annurev.bi.57.070188.004203

Higashi, D. L., Biais, N., Weyand, N. J., Agellon, A., Sisko, J. L., Brown, L. M., & So, M. (2011). N. elongata produces type IV pili that mediate interspecies gene transfer with N. gonorrhoeae. *PloS One, 6*(6), e21373. doi:10.1371/journal.pone.0021373

Hill, D. J., Griffiths, N. J., Borodina, E., & Virji, M. (2010). Cellular and molecular biology of *Neisseria meningitidis* colonization and invasive disease. *Clinical Science (London, England: 1979), 118*(9), 547-564. doi:10.1042/cs20090513

Hook-Barnard, I. G., & Hinton, D. M. (2007). Transcription Initiation by Mix and Match Elements: Flexibility for Polymerase Binding to Bacterial Promoters. *Gene Regulation and Systems Biology, 1*, 275-293.

Hoover, T. R., Santero, E., Porter, S., & Kustu, S. (1990). The integration host factor stimulates interaction of RNA polymerase with NIFA, the transcriptional activator for nitrogen fixation operons. *Cell, 63*(1), 11-22.

Hopman, C. T. P., Speijer, D., van der Ende, A., & Pannekoek, Y. (2010). Identification of a novel anti-σE factor in *Neisseria meningitidis*. *BMC Microbiology, 10*(1), 164. doi:10.1186/1471-2180-10-164

Hsieh, M., & Gralla, J. D. (1994). Analysis of the N-terminal leucine heptad and hexad repeats of sigma 54. *J Mol Biol, 239*(1), 15-24. doi:10.1006/jmbi.1994.1347

Huerta, A. M., & Collado-Vides, J. (2003). Sigma70 promoters in *Escherichia coli*: specific transcription in dense regions of overlapping promoter-like signals. *J Mol Biol, 333*(2), 261-278.

Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M. C., . . . Bork, P. (2016). eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Research, 44*(D1), D286-293. doi:10.1093/nar/gkv1248

Huis in 't Veld, R. A., Willemsen, A. M., van Kampen, A. H., Bradley, E. J., Baas, F., Pannekoek, Y., & van der Ende, A. (2011). Deep sequencing whole transcriptome exploration of the sigmaE regulon in *Neisseria meningitidis*. *PloS One, 6*(12), e29002. doi:10.1371/journal.pone.0029002

Huis in ‘t Veld, R. A. G., Kramer, G., van der Ende, A., Speijer, D., & Pannekoek, Y. (2017). The Hfq regulon of *Neisseria meningitidis*. *FEBS Open Bio, 7*(6), 777-788. doi:10.1002/2211-5463.12218

Hurwitz, J., Furth, J. J., Anders, M., Ortiz, P. J., & August, J. T. (1961). The Enzymatic Incorporation of Ribonucleotides into RNA and the Role of DNA. *26*, 91-100. doi:10.1101/sqb.1961.026.01.014

Ieva, R., Alaimo, C., Delany, I., Spohn, G., Rappuoli, R., & Scarlato, V. (2005). CrgA Is an Inducible LysR-Type Regulator of *Neisseria meningitidis*, Acting both as a Repressor and as an Activator of Gene Transcription. *Journal of Bacteriology, 187*(10), 3421-3430. doi:10.1128/jb.187.10.3421-3430.2005

Imhaus, A. F., & Duménil, G. (2014). The number of *Neisseria meningitidis* type IV pili determines host cell interaction. *The EMBO Journal, 33*(16), 1767.

Inda, M. E., Vandenbranden, M., Fernández, A., de Mendoza, D., Ruysschaert, J.-M., & Cybulski, L. E. (2014). A lipid-mediated conformational switch modulates the thermosensing activity of DesK. *Proceedings of the National Academy of Sciences, 111*(9), 3579.

Ishihama, A. (2010). Prokaryotic genome regulation: multifactor promoters, multitarget regulators and hierarchic networks. *FEMS Microbiology Reviews, 34*(5), 628-645. doi:10.1111/j.1574-6976.2010.00227.x

Ishimoto, K. S., & Lory, S. (1992). Identification of pilR, which encodes a transcriptional activator of the *Pseudomonas aeruginosa* pilin gene. *J Bacteriol, 174*(11), 3514-3521.

Jamet, A., Euphrasie, D., Martin, P., & Nassif, X. (2013). Identification of Genes Involved in *Neisseria meningitidis* Colonization. *Infect Immun, 81*(9), 3375-3381. doi:10.1128/IAI.00421-13

Jamet, A., Jousset, A. B., Euphrasie, D., Mukorako, P., Boucharlat, A., Ducousso, A., . . . Nassif, X. (2015). A New Family of Secreted Toxins in Pathogenic Neisseria Species. *PLoS Pathogens, 11*(1), e1004592. doi:10.1371/journal.ppat.1004592

Janaszak, A., Nadratowska-Wesołowska, B., Konopa, G., & Taylor, A. (2009). The P1 promoter of the *Escherichia coli rpoH* gene is utilized by σ70-RNAP or σS-RNAP depending on growth phase. *FEMS Microbiology Letters, 291*(1), 65-72. doi:10.1111/j.1574-6968.2008.01436.x

Jarva, H., Ram, S., Vogel, U., Blom, A. M., & Meri, S. (2005). Binding of the complement inhibitor C4bp to serogroup B *Neisseria meningitidis*. *J Immunol, 174*(10), 6299-6307.

Jarvis, G. A., & Vedros, N. A. (1987). Sialic acid of group B *Neisseria meningitidis* regulates alternative complement pathway activation. *Infect Immun, 55*(1), 174.

Jeon, Y. H., Negishi, T., Shirakawa, M., Yamazaki, T., Fujita, N., Ishihama, A., & Kyogoku, Y. (1995). Solution structure of the activator contact domain of the RNA polymerase alpha subunit. *Science, 270*(5241), 1495-1497.

Jin, S., Ishimoto, K. S., & Lory, S. (1994). PilR, a transcriptional regulator of piliation in Pseudomonas aeruginosa, binds to a cis-acting sequence upstream of the pilin gene promoter. *Molecular Microbiology, 14*(5), 1049-1057.

Jishage, M., & Ishihama, A. (1995). Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of sigma 70 and sigma 38. *Journal of Bacteriology, 177*(23), 6832-6835. doi:10.1128/jb.177.23.6832-6835.1995

Jishage, M., & Ishihama, A. (1999). Transcriptional organization and in vivo role of the *Escherichia coli* rsd gene, encoding the regulator of RNA polymerase sigma D. *J Bacteriol, 181*(12), 3768-3776.

Jishage, M., Iwata, A., Ueda, S., & Ishihama, A. (1996). Regulation of RNA polymerase sigma subunit synthesis in *Escherichia coli*: intracellular levels of four species of sigma subunit under various growth conditions. *Journal of Bacteriology, 178*(18), 5447-5451.

Jolley, K. A., & Maiden, M. C. J. (2010). BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics, 11*(1), 595. doi:10.1186/1471-2105-11-595

Jordan, P. W., & Saunders, N. J. (2009). Host Iron Binding Proteins Acting as Niche Indicators for *Neisseria meningitidis*. *PLoS One, 4*(4), e5198. doi:10.1371/journal.pone.0005198

Jovanovic, G., & Model, P. (1997). PspF and IHF bind co-operatively in the psp promoter-regulatory region of *Escherichia coli*. *Molecular Microbiology, 25*(3), 473-481.

Kahler, C. M., Martin, L. E., Shih, G. C., Rahman, M. M., Carlson, R. W., & Stephens, D. S. (1998). The (alpha2-->8)-linked polysialic acid capsule and lipooligosaccharide structure both contribute to the ability of serogroup B *Neisseria meningitidis* to resist the bactericidal activity of normal human serum. *Infect Immun, 66*(12), 5939-5947.

Kallstrom, H., Liszewski, M. K., Atkinson, J. P., & Jonsson, A. B. (1997). Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic *Neisseria*. *Mol Microbiol, 25*(4), 639-647.

Keck, T., Leiacker, R., Riechelmann, H., & Rettinger, G. (2000). Temperature Profile in the Nasal Cavity. *The Laryngoscope, 110*(4), 651-654. doi:10.1097/00005537-200004000-00021

Keilty, S., & Rosenberg, M. (1987). Constitutive function of a positively regulated promoter reveals new sequences essential for activity. *Journal of Biological Chemistry, 262*(13), 6389-6395.

Keseler, I. M., & Kaiser, D. (1995). An early A-signal-dependent gene in *Myxococcus xanthus* has a sigma 54-like promoter. *Journal of Bacteriology, 177*(16), 4638-4644.

King, T., Lucchini, S., Hinton, J. C. D., & Gobius, K. (2010). Transcriptomic Analysis of *Escherichia coli* O157:H7 and K-12 Cultures Exposed to Inorganic and Organic Acids in Stationary Phase Reveals Acidulant- and Strain-Specific Acid Tolerance Responses. *Appl Environ Microbiol, 76*(19), 6514-6528. doi:10.1128/AEM.02392-09

Kirchner, M., Heuer, D., & Meyer, T. F. (2005). CD46-independent binding of neisserial type IV pili and the major pilus adhesin, PilC, to human epithelial cells. *Infect Immun, 73*(5), 3072-3082. doi:10.1128/iai.73.5.3072-3082.2005

Klein, G., Stupak, A., Biernacka, D., Wojtkiewicz, P., Lindner, B., & Raina, S. (2016). Multiple Transcriptional Factors Regulate Transcription of the rpoE Gene in *Escherichia coli* under Different Growth Conditions and When the Lipopolysaccharide Biosynthesis Is Defective. *291*(44), 22999-23019. doi:10.1074/jbc.M116.748954

Kolappan, S., Coureuil, M., Yu, X., Nassif, X., Egelman, E. H., & Craig, L. (2016). Structure of the *Neisseria meningitidis* Type IV pilus. *Nature Communications, 7*, 13015. doi:10.1038/ncomms13015
https://www.nature.com/articles/ncomms13015#supplementary-information

Koo, B.-M., Rhodius, V. A., Nonaka, G., deHaseth, P. L., & Gross, C. A. (2009). Reduced capacity of alternative sigmas to melt promoters ensures stringent promoter recognition. *Genes & Development, 23*(20), 2426-2436. doi:10.1101/gad.1843709

Kortmann, J., & Narberhaus, F. (2012). Bacterial RNA thermometers: molecular zippers and switches. *Nature Reviews Microbiology, 10*, 255. doi:10.1038/nrmicro2730 https://www.nature.com/articles/nrmicro2730#supplementary-information

Kulshin, V. A., Zähringer, U., Lindner, B., Frasch, C. E., Tsai, C. M., Dmitriev, B. A., & Rietschel, E. T. (1992). Structural characterization of the lipid A component of pathogenic *Neisseria meningitidis*. *Journal of Bacteriology, 174*(6), 1793.

Kumar, A., Williamson, H. S., Fujita, N., Ishihama, A., & Hayward, R. S. (1995). A partially functional 245-amino-acid internal deletion derivative of *Escherichia coli* sigma 70. *Journal of Bacteriology, 177*(17), 5193-5196.

Kuwae, A., Sjölinder, H., Eriksson, J., Eriksson, S., Chen, Y., & Jonsson, A.-B. (2011). NafA Negatively Controls *Neisseria meningitidis* Piliation. *PloS One, 6*(7), e21749. doi:10.1371/journal.pone.0021749

LaForce, M. F., Ravenscroft, N., Djingarey, M., & Viviani, S. (2009). Epidemic meningitis due to Group A *Neisseria meningitidis* in the African meningitis belt: a persistent problem with an imminent solution. *Vaccine, 27 Suppl 2*, B13-19. doi:10.1016/j.vaccine.2009.04.062

Lange, R., & Hengge-Aronis, R. (1991). Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol Microbiol, 5*(1), 49-59. doi:10.1111/j.1365-2958.1991.tb01825.x

Lappann, M., Otto, A., Brauer, M., Becher, D., Vogel, U., & Johswich, K. (2016). Impact of Moderate Temperature Changes on *Neisseria meningitidis* Adhesion Phenotypes and Proteome. *Infection and Immunity, 84*(12), 3484-3495. doi:10.1128/iai.00584-16

Larribe, M., Taha, M. K., Topilko, A., & Marchal, C. (1997). Control of *Neisseria gonorrhoeae* pilin gene expression by environmental factors: involvement of the pilA/pilB regulatory genes. *Microbiology, 143 (Pt 5)*, 1757-1764. doi:10.1099/00221287-143-5-1757

Laskos, L., Dillard, J. P., Seifert, H. S., Fyfe, J. A., & Davies, J. K. (1998). The pathogenic neisseriae contain an inactive rpoN gene and do not utilize the pilE sigma54 promoter. *Gene, 208*(1), 95-102.

Laskos, L., Ryan, C. S., Fyfe, J. A., & Davies, J. K. (2004). The RpoH-mediated stress response in *Neisseria gonorrhoeae* is regulated at the level of activity. *Journal of Bacteriology, 186*(24), 8443-8452. doi:10.1128/jb.186.24.8443-8452.2004

Laver, J. R., Hughes, S. E., & Read, R. C. (2015). Chapter Three - Neisserial Molecular Adaptations to the Nasopharyngeal Niche. In R. K. Poole (Ed.), *Advances in Microbial Physiology* (Vol. 66, pp. 323-355): Academic Press.

Lécuyer, H., Borgel, D., Nassif, X., & Coureuil, M. (2017). Pathogenesis of meningococcal purpura fulminans. *Pathogens and Disease, 75*(3), ftx027-ftx027. doi:10.1093/femspd/ftx027

Lecuyer, H., Nassif, X., & Coureuil, M. (2012). Two strikingly different signaling pathways are induced by meningococcal type IV pili on endothelial and epithelial cells. *Infect Immun, 80*(1), 175-186. doi:10.1128/iai.05837-11

Lee, J., & Borukhov, S. (2016). Bacterial RNA Polymerase-DNA Interaction-The Driving Force of Gene Expression and the Target for Drug Action. *Frontiers in molecular biosciences, 3*, 73-73. doi:10.3389/fmolb.2016.00073

Lee, S. J., & Gralla, J. D. (2001). Sigma38 (rpoS) RNA Polymerase Promoter Engagement via −10 Region Nucleotides. *276*(32), 30064-30071. doi:10.1074/jbc.M102886200

Leibman, M., & Hochschild, A. (2007). A σ-core interaction of the RNA polymerase holoenzyme that enhances promoter escape. *The EMBO Journal, 26*(6), 1579-1590. doi:10.1038/sj.emboj.7601612

Lévi-Meyrueis, C., Monteil, V., Sismeiro, O., Dillies, M.-A., Kolb, A., Monot, M., . . . Norel, F. (2015). Repressor activity of the RpoS/σ(S)-dependent RNA polymerase requires DNA binding. *Nucleic Acids Research, 43*(3), 1456-1468. doi:10.1093/nar/gku1379

Lewis, L. A., & Ram, S. (2014). Meningococcal disease and the complement system. *Virulence, 5*(1), 98-126. doi:10.4161/viru.26515

Li, J., Passaglia, L., Rombel, I., Yan, D., & Kustu, S. (1999). Mutations Affecting Motifs of Unknown Function in the Central Domain of Nitrogen Regulatory Protein C. *Journal of Bacteriology, 181*(17), 5443.

Lim, B., Miyazaki, R., Neher, S., Siegele, D. A., Ito, K., Walter, P., . . . Gross, C. A. (2013). Heat shock transcription factor sigma32 co-opts the signal recognition particle to regulate protein homeostasis in E. coli. *PLoS Biol, 11*(12), e1001735. doi:10.1371/journal.pbio.1001735

Link, A. J., Phillips, D., & Church, G. M. (1997). Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J Bacteriol, 179*(20), 6228-6237.

Liu, G., Tang, C. M., & Exley, R. M. (2015). Non-pathogenic *Neisseria*: members of an abundant, multi-habitat, diverse genus. *Microbiology, 161*(7), 1297-1312. doi:doi:10.1099/mic.0.000086

Liu, S. V., Saunders, N. J., Jeffries, A., & Rest, R. F. (2002). Genome Analysis and Strain Comparison of Correia Repeats and Correia Repeat-Enclosed Elements in Pathogenic *Neisseria*. *Journal of Bacteriology, 184*(22), 6163-6173. doi:10.1128/JB.184.22.6163-6173.2002

Liu, T.Y., Gotschlich, E. C., Jonssen, E. K., & Wysocki, J. R. (1971). Studies on the Meningococcal Polysaccharides: i. Composition and chemical properties of the group a polysaccharide. *246*(9), 2849-2858.

Loh, E., Kugelberg, E., Tracy, A., Zhang, Q., Gollan, B., Ewles, H., . . . Tang, C. M. (2013). Temperature triggers immune evasion by *Neisseria meningitidis*. *Nature, 502*(7470), 237-240. doi:10.1038/nature12616

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology, 15*(12), 550. doi:10.1186/s13059-014-0550-8

Maiden, M. C. J., Jansen van Rensburg, M. J., Bray, J. E., Earle, S. G., Ford, S. A., Jolley, K. A., & McCarthy, N. D. (2013). MLST revisited: the gene-by-gene approach to bacterial genomics. *Nature reviews. Microbiology, 11*(10), 728-736. doi:10.1038/nrmicro3093

Marri, P. R., Paniscus, M., Weyand, N. J., Rendon, M. A., Calton, C. M., Hernandez, D. R., . . . So, M. (2010). Genome sequencing reveals widespread virulence gene exchange among human *Neisseria* species. *PloS One, 5*(7), e11835. doi:10.1371/journal.pone.0011835

Mauri, M., & Klumpp, S. (2014). A Model for Sigma Factor Competition in Bacterial Cells. *PLoS Computational Biology, 10*(10), e1003845. doi:10.1371/journal.pcbi.1003845

McClure, R., Tjaden, B., & Genco, C. (2014). Identification of sRNAs expressed by the human pathogen *Neisseria gonorrhoeae* under disparate growth conditions. *5*(456). doi:10.3389/fmicb.2014.00456

Mekler, V., Kortkhonjia, E., Mukhopadhyay, J., Knight, J., Revyakin, A., Kapanidis, A. N., . . . Ebright, R. H. (2002). Structural organization of bacterial RNA polymerase holoenzyme and the RNA polymerase-promoter open complex. *Cell, 108*(5), 599-614.

Melican, K., & Dumenil, G. (2012). Vascular colonization by *Neisseria meningitidis*. *Current Opinion in Microbiology, 15*(1), 50-56. doi:10.1016/j.mib.2011.10.008

Merrick, M., & Chambers, S. (1992). The helix-turn-helix motif of sigma 54 is involved in recognition of the -13 promoter region. *Journal of Bacteriology, 174*(22), 7221-7226.

Merz, A. J., So, M., & Sheetz, M. P. (2000). Pilus retraction powers bacterial twitching motility. *Nature, 407*(6800), 98-102. doi:10.1038/35024105

Mikaty, G., Soyer, M., Mairey, E., Henry, N., Dyer, D., Forest, K. T., . . . Dumenil, G. (2009). Extracellular bacterial pathogen induces host cell surface reorganization to resist shear stress. *PLoS Pathogens, 5*(2), e1000314. doi:10.1371/journal.ppat.1000314

Miller, F., Phan, G., Brissac, T., Bouchiat, C., Lioux, G., Nassif, X., & Coureuil, M. (2014). The Hypervariable Region of Meningococcal Major Pilin PilE Controls the Host Cell Response via Antigenic Variation. *MBio, 5*(1).

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor; NY: Cold Spring Harbor Laboratory Press.

Miyazaki, R., Yura, T., Suzuki, T., Dohmae, N., Mori, H., & Akiyama, Y. (2016). A Novel SRP Recognition Sequence in the Homeostatic Control Region of Heat Shock Transcription Factor sigma32. *Sci Rep, 6*, 24147. doi:10.1038/srep24147

Morand, P. C., Bille, E., Morelle, S., Eugène, E., Beretti, J. L., Wolfgang, M., . . . Nassif, X. (2004). Type IV pilus retraction in pathogenic *Neisseria* is regulated by the PilC proteins. *The EMBO Journal, 23*(9), 2009.

Morand, P. C., Tattevin, P., Eugene, E., Beretti, J. L., & Nassif, X. (2001). The adhesive property of the type IV pilus-associated component PilC1 of pathogenic *Neisseria* is supported by the conformational structure of the N-terminal part of the molecule. *Molecular Microbiology, 40*(4), 846-856.

Morett, E., & Buck, M. (1989). In vivo studies on the interaction of RNA polymerase-sigma 54 with the Klebsiella pneumoniae and Rhizobium meliloti *nifH* promoters. The role of NifA in the formation of an open promoter complex. *J Mol Biol, 210*(1), 65-77.

Murakami, K. S. (2013). X-ray crystal structure of *Escherichia coli* RNA polymerase sigma70 holoenzyme. *J Biol Chem, 288*(13), 9126-9134. doi:10.1074/jbc.M112.430900

Murakami, K. S., Masuda, S., Campbell, E. A., Muzzin, O., & Darst, S. A. (2002). Structural Basis of Transcription Initiation: An RNA Polymerase Holoenzyme-DNA Complex. *Science, 296*(5571), 1285.

Myers, S. A., Wright, J., Peckner, R., Kalish, B. T., Zhang, F., & Carr, S. A. (2018). Discovery of proteins associated with a predefined genomic locus *via* dCas9–APEX-mediated proximity labeling. *Nature Methods*. doi:10.1038/s41592-018-0007-1

Nagai, H., Yano, R., Erickson, J. W., & Yura, T. (1990). Transcriptional regulation of the heat shock regulatory gene *rpoH* in *Escherichia coli*: involvement of a novel catabolite-sensitive promoter. *Journal of Bacteriology, 172*(5), 2710.

Nakahigashi, K., Yanagi, H., & Yura, T. (1995). Isolation and sequence analysis of rpoH genes encoding sigma 32 homologs from gram negative bacteria: conserved mRNA and protein segments for heat shock regulation. *Nucleic Acids Research, 23*(21), 4383-4390.

Nassif, X., Beretti, J. L., Lowy, J., Stenberg, P., O'Gaora, P., Pfeifer, J., . . . So, M. (1994). Roles of pilin and PilC in adhesion of *Neisseria meningitidis* to human epithelial and endothelial cells. *Proc Natl Acad Sci U S A, 91*(9), 3769-3773.

Nassif, X., Lowy, J., Stenberg, P., O'Gaora, P., Ganji, A., & So, M. (1993). Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. *Mol Microbiol, 8*(4), 719-725.

Nathan, N., Rose, A. M. C., Legros, D., Tiendrebeogo, S. R. M., Bachy, C., Bjørløw, E., . . . Caugant, D. A. (2007). Meningitis Serogroup W135 Outbreak, Burkina Faso, 2002. *Emerging Infectious Diseases, 13*(6), 920-923. doi:10.3201/eid1306.060940

Nawaz, T., Hardy, D. J., & Bonnez, W. (1996). *Neisseria elongata* subsp. elongata, a case of human endocarditis complicated by pseudoaneurysm. *Journal of Clinical Microbiology, 34*(3), 756.

Nickels, B. E., Garrity, S. J., Mekler, V., Minakhin, L., Severinov, K., Ebright, R. H., & Hochschild, A. (2005). The interaction between σ70 and the β-flap of *Escherichia coli*; RNA polymerase inhibits extension of nascent RNA during early elongation. *Proc Natl Acad Sci U S A, 102*(12), 4488.

Noble, R. C., Cooper, R. M., & Miller, B. R. (1979). Pharyngeal colonisation by *Neisseria gonorrhoeae* and *Neisseria meningitidis* in black and white patients attending a venereal disease clinic. *British Journal of Venereal Diseases, 55*(1), 14-19.

Nonaka, G., Blankschien, M., Herman, C., Gross, C. A., & Rhodius, V. A. (2006). Regulon and promoter analysis of the E. coli heat-shock factor, σ(32), reveals a multifaceted cellular response to heat stress. *Genes and Development, 20*(13), 1776-1789. doi:10.1101/gad.1428206

Obergfell, K. P., & Seifert, H. S. (2016). The Pilin N-terminal Domain Maintains *Neisseria gonorrhoeae* Transformation Competence during Pilus Phase Variation. *PLOS Genetics, 12*(5), e1006069. doi:10.1371/journal.pgen.1006069

Obrist, M., & Narberhaus, F. (2005). Identification of a Turnover Element in Region 2.1 of *Escherichia coli* σ32 by a Bacterial One-Hybrid Approach. *187*(11), 3807-3813. doi:10.1128/jb.187.11.3807-3813.2005

Österberg, S., Peso-Santos, T. d., & Shingler, V. (2011). Regulation of Alternative Sigma Factor Use. *Annual Review of Microbiology, 65*(1), 37-55. doi:doi:10.1146/annurev.micro.112408.134219

Pabo, C. O., & Sauer, R. T. (1992). Transcription factors: structural families and principles of DNA recognition. *Annu Rev Biochem, 61*, 1053-1095. doi:10.1146/annurev.bi.61.070192.005201

Paget, M. S. (2015). Bacterial Sigma Factors and Anti-Sigma Factors: Structure, Function and Distribution. *Biomolecules, 5*(3), 1245-1265. doi:10.3390/biom5031245

Pannekoek, Y., Huis in ‘t Veld, R., Hopman, C. T. P., Langerak, A. A. J., Speijer, D., & van der Ende, A. (2009). Molecular characterization and identification of proteins regulated by Hfq in *Neisseria meningitidis*. *FEMS Microbiology Letters, 294*(2), 216-224. doi:10.1111/j.1574-6968.2009.01568.x

Pathan, N., Faust, S. N., & Levin, M. (2003). Pathophysiology of meningococcal meningitis and septicaemia. *Archives of Disease in Childhood, 88*(7), 601.

Pelicic, V., Morelle, S., Lampe, D., & Nassif, X. (2000). Mutagenesis of *Neisseria meningitidis* by In Vitro Transposition of Himar1 mariner. *Journal of Bacteriology, 182*(19), 5391.

Pelton, J. G., Kustu, S., & Wemmer, D. E. (1999). Solution structure of the DNA-binding domain of NtrC with three alanine substitutions. *J Mol Biol, 292*(5), 1095-1110. doi:10.1006/jmbi.1999.3140

Periago, P. M., van Schaik, W., Abee, T., & Wouters, J. A. (2002). Identification of proteins involved in the heat stress response of *Bacillus cereus* ATCC 14579. *Appl Environ Microbiol, 68*(7), 3486-3495.

Perkins-Balding, D., Ratliff-Griffin, M., & Stojiljkovic, I. (2004). Iron Transport Systems in *Neisseria meningitidis*. *Microbiology and Molecular Biology Reviews, 68*(1), 154-171. doi:10.1128/MMBR.68.1.154-171.2004

Piper, S. E., Mitchell, J. E., Lee, D. J., & Busby, S. J. W. (2009). A global view of *Escherichia coli* Rsd protein and its interactions. *Molecular BioSystems, 5*(12), 1943-1947. doi:10.1039/B904955J

Pizza, M., & Rappuoli, R. (2015). *Neisseria meningitidis*: pathogenesis and immunity. *Current Opinion in Microbiology, 23*, 68-72. doi:https://doi.org/10.1016/j.mib.2014.11.006

Popham, D. L., Szeto, D., Keener, J., & Kustu, S. (1989). Function of a bacterial activator protein that binds to transcriptional enhancers. *Science, 243*(4891), 629.

Power, P. M., Seib, K. L., & Jennings, M. P. (2006). Pilin glycosylation in *Neisseria meningitidis* occurs by a similar pathway to wzy-dependent O-antigen biosynthesis in *Escherichia coli*. *Biochem Biophys Res Commun, 347*(4), 904-908. doi:10.1016/j.bbrc.2006.06.182

Pribnow, D. (1975). Bacteriophage T7 early promoters: Nucleotide sequences of two RNA polymerase binding sites. *Journal of molecular biology, 99*(3), 419-443. doi:https://doi.org/10.1016/S0022-2836(75)80136-7

Qi, H., Menzel, R., & Tse-Dinh, Y. C. (1996). Effect of the deletion of the sigma 32-dependent promoter (P1) of the *Escherichia coli* topoisomerase I gene on thermotolerance. *Mol Microbiol, 21*(4), 703-711.

Raffaelle, M., Kanin, E. I., Vogt, J., Burgess, R. R., & Ansari, A. Z. (2005). Holoenzyme Switching and Stochastic Release of Sigma Factors from RNA Polymerase In Vivo. *Molecular Cell, 20*(3), 357-366. doi:https://doi.org/10.1016/j.molcel.2005.10.011

Ramsey, M. E., Hackett, K. T., Kotha, C., & Dillard, J. P. (2012). New complementation constructs for inducible and constitutive gene expression in *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Applied and Environmental Microbiology, 78*(9), 3068-3078. doi:10.1128/aem.07871-11

Reitzer, L. J., Bueno, R., Cheng, W. D., Abrams, S. A., Rothstein, D. M., Hunt, T. P., . . . Magasanik, B. (1987). Mutations that create new promoters suppress the sigma 54 dependence of glnA transcription in *Escherichia coli*. *Journal of Bacteriology, 169*(9), 4279-4284.

Remmele, C. W., Xian, Y., Albrecht, M., Faulstich, M., Fraunholz, M., Heinrichs, E., . . . Rudel, T. (2014). Transcriptional landscape and essential genes of *Neisseria gonorrhoeae*. *Nucleic Acids Research, 42*(16), 10579-10595. doi:10.1093/nar/gku762

Rendon, M. A., Hockenberry, A. M., McManus, S. A., & So, M. (2013). Sigma factor RpoN (sigma54) regulates *pilE* transcription in commensal *Neisseria elongata*. *Molecular Microbiology, 90*(1), 103-113. doi:10.1111/mmi.12350

Rendón, M. A., Lona, B., Ma, M., & So, M. (2018). RpoN and the Nps and Npa two-component regulatory system control *pilE* transcription in commensal *Neisseria*. *MicrobiologyOpen, 0*(0), e00713. doi:doi:10.1002/mbo3.713

Rhodius, V. A., & Mutalik, V. K. (2010). Predicting strength and function for promoters of the *Escherichia coli* alternative sigma factor, σE. *Proceedings of the National Academy of Sciences, 107*(7), 2854-2859. doi:10.1073/pnas.0915066107

Rhodius, V. A., Suh, W. C., Nonaka, G., West, J., & Gross, C. A. (2005). Conserved and Variable Functions of the σE Stress Response in Related Genomes. *PLoS Biology, 4*(1), e2. doi:10.1371/journal.pbio.0040002

Richardson, A. R., & Stojiljkovic, I. (1999). HmbR, a Hemoglobin-Binding Outer Membrane Protein of *Neisseria meningitidis*, Undergoes Phase Variation. *Journal of Bacteriology, 181*(7), 2067-2074.

Roncarati, D., & Scarlato, V. (2017). Regulation of heat-shock genes in bacteria: from signal sensing to gene expression output. *FEMS Microbiology Reviews, 41*(4), 549-574. doi:10.1093/femsre/fux015

Rosenstein, N. E., Perkins, B. A., Stephens, D. S., Popovic, T., & Hughes, J. M. (2001). Meningococcal disease. *New England Journal of Medicine, 344*(18), 1378-1388. doi:10.1056/nejm200105033441807

Ross, W., Vrentas, Catherine E., Sanchez-Vazquez, P., Gaal, T., & Gourse, Richard L. (2013). The Magic Spot: A ppGpp Binding Site on *E. coli* RNA Polymerase Responsible for Regulation of Transcription Initiation. *Molecular Cell, 50*(3), 420-429. doi:https://doi.org/10.1016/j.molcel.2013.03.021

Rytkönen, A., Albiger, B., Hansson-Palo, P., Källström, H., Olcén, P., Fredlund, H., & Jonsson, A.B. (2004). *Neisseria meningitidis* Undergoes PilC Phase Variation and PilE Sequence Variation during Invasive Disease. *The Journal of Infectious Diseases, 189*(3), 402-409. doi:10.1086/381271

Saecker, R. M., Record, M. T., & deHaseth, P. L. (2011). Mechanism of Bacterial Transcription Initiation: RNA Polymerase - Promoter Binding, Isomerization to

Initiation-Competent Open Complexes, and Initiation of RNA Synthesis. *Journal of Molecular Biology, 412*(5), 754-771. doi:https://doi.org/10.1016/j.jmb.2011.01.018

Sarkari, J., Pandit, N., Moxon, E. R., & Achtman, M. (1994). Variable expression of the Opc outer membrane protein in *Neisseria meningitidis* is caused by size variation of a promoter containing poly-cytidine. *Mol Microbiol, 13*(2), 207-217. doi:10.1111/j.1365-2958.1994.tb00416.x

Schaefer, J., Engl, C., Zhang, N., Lawton, E., & Buck, M. (2015). Genome wide interactions of wild-type and activator bypass forms of σ54. *Nucleic Acids Research, 43*(15), 7280-7291. doi:10.1093/nar/gkv597

Schneider, M. C., Prosser, B. E., Caesar, J. J., Kugelberg, E., Li, S., Zhang, Q., . . . Lea, S. M. (2009). *Neisseria meningitidis* recruits factor H using protein mimicry of host carbohydrates. *Nature, 458*(7240), 890-893. doi:10.1038/nature07769

Schoen, C., Tettelin, H., Parkhill, J., & Frosch, M. (2009). Genome flexibility in *Neisseria meningitidis*. *Vaccine, 27*, B103-B111. doi:https://doi.org/10.1016/j.vaccine.2009.04.064

Sengupta, P., & Garrity, P. (2013). Sensing temperature. *Current Biology, 23*(8), R304-R307. doi:10.1016/j.cub.2013.03.009

Severinov, K., Mooney, R., Darst, S. A., & Landick, R. (1997). Tethering of the large subunits of *Escherichia coli* RNA polymerase. *J Biol Chem, 272*(39), 24137-24140.

Severinova, E., Severinov, K., Fenyö, D., Marr, M., Brody, E. N., Roberts, J. W., . . . Darst, S. A. (1996). Domain Organization of the *Escherichia coli* RNA Polymerase σ70 Subunit. *Journal of molecular biology, 263*(5), 637-647. doi:https://doi.org/10.1006/jmbi.1996.0604

Sharma, C. M., Hoffmann, S., Darfeuille, F., Reignier, J., Findeiss, S., Sittka, A., . . . Vogel, J. (2010). The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature, 464*(7286), 250-255. doi:10.1038/nature08756

Sharma, U. K., & Chatterji, D. (2010). Transcriptional switching in *Escherichia coli* during stress and starvation by modulation of sigma activity. *FEMS Microbiology Reviews, 34*(5), 646-657. doi:10.1111/j.1574-6976.2010.00223.x

Simon, R., Priefer, U., & Pühler, A. (1983). A Broad Host Range Mobilization System for In Vivo Genetic Engineering: Transposon Mutagenesis in Gram Negative Bacteria. *Bio/Technology, 1*, 784. doi:10.1038/nbt1183-784

Spinosa, M. R., Progida, C., Tala, A., Cogli, L., Alifano, P., & Bucci, C. (2007). The *Neisseria meningitidis* capsule is important for intracellular survival in human cells. *Infect Immun, 75*(7), 3594-3603. doi:10.1128/iai.01945-06

Stephens, D. S., Greenwood, B., & Brandtzaeg, P. (2007). Epidemic meningitis, meningococcaemia, and *Neisseria meningitidis*. *The Lancet, 369*(9580), 2196-2210. doi:10.1016/S0140-6736(07)61016-2

Storz, G., Vogel, J., & Wassarman, K. M. (2011). Regulation by Small RNAs in Bacteria: Expanding Frontiers. *Molecular Cell, 43*(6), 880-891. doi:10.1016/j.molcel.2011.08.022

Straus, D. B., Walter, W. A., & Gross, C. A. (1987). The heat shock response of E. coli is regulated by changes in the concentration of sigma 32. *Nature, 329*(6137), 348-351. doi:10.1038/329348a0

Studholme, D. J., & Buck, M. (2000). The biology of enhancer-dependent transcriptional regulation in bacteria: insights from genome sequences. *FEMS Microbiology Letters, 186*(1), 1-9.

Swartley, J. S., Marfin, A. A., Edupuganti, S., Liu, L. J., Cieslak, P., Perkins, B., . . . Stephens, D. S. (1997). Capsule switching of *Neisseria meningitidis*. *Proc Natl Acad Sci U S A, 94*(1), 271-276.

Taha, M. K., Giorgini, D., & Nassif, X. (1996). The pilA regulatory gene modulates the pilus-mediated adhesion of *Neisseria meningitidis* by controlling the transcription of pilC1. *Molecular Microbiology, 19*(5), 1073-1084.

Takahashi, H., Yanagisawa, T., Kim, K. S., Yokoyama, S., & Ohnishi, M. (2012). Meningococcal PilV Potentiates *Neisseria meningitidis* Type IV Pilus-Mediated Internalization into Human Endothelial and Epithelial Cells. *Infect Immun, 80*(12), 4154.

Tam, C., Collinet, B., Lau, G., Raina, S., & Missiakas, D. (2002). Interaction of the Conserved Region 4.2 of ςE with the RseA Anti-sigma Factor. *277*(30), 27282-27287. doi:10.1074/jbc.M202881200

Tan, F. Y., Wormann, M. E., Loh, E., Tang, C. M., & Exley, R. M. (2015). Characterization of a novel antisense RNA in the major pilin locus of *Neisseria meningitidis* influencing antigenic variation. *Journal of Bacteriology, 197*(10), 1757-1768. doi:10.1128/jb.00082-15

Tang, G. Q., Bandwar, R. P., & Patel, S. S. (2005). Extended upstream A-T sequence increases T7 promoter strength. *J Biol Chem, 280*(49), 40707-40713. doi:10.1074/jbc.M508013200

Taylor, M., Butler, R., Chambers, S., Casimiro, M., Badii, F., & Merrick, M. (1996). The RpoN-box motif of the RNA polymerase sigma factor sigma N plays a role in promoter recognition. *Molecular Microbiology, 22*(5), 1045-1054.

Taylor, M., Butler, R., Chambers, S., Casimiro, M., Badii, F., & Merrick, M. (1996). The RpoN-box motif of the RNA polymerase sigma factor σN plays a role in promoter recognition. *Molecular Microbiology, 22*(5), 1045-1054. doi:10.1046/j.1365-2958.1996.01547.x

Tettelin, H., Saunders, N. J., Heidelberg, J., Jeffries, A. C., Nelson, K. E., Eisen, J. A., . . . Venter, J. C. (2000). Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. *Science, 287*(5459), 1809-1815.

Tilly, K., Erickson, J., Sharma, S., & Georgopoulos, C. (1986). Heat shock regulatory gene rpoH mRNA level increases after heat shock in *Escherichia coli*. *Journal of Bacteriology, 168*(3), 1155-1158.

Tobiason, D. M., & Seifert, H. S. (2001). Inverse relationship between pilus-mediated gonococcal adherence and surface expression of the pilus receptor, CD46. *Microbiology, 147*(Pt 8), 2333-2340. doi:10.1099/00221287-147-8-2333

Traore, Y., Njanpop-Lafourcade, B. M., Adjogble, K. L., Lourd, M., Yaro, S., Nacro, B., . . . Gessner, B. D. (2006). The rise and fall of epidemic *Neisseria meningitidis* serogroup W135 meningitis in Burkina Faso, 2002-2005. *Clin Infect Dis, 43*(7), 817-822. doi:10.1086/507339

Treangen, T. J., Ambur, O. H., Tonjum, T., & Rocha, E. P. C. (2008). The impact of the neisserial DNA uptake sequences on genome evolution and stability. *Genome Biology, 9*(3), R60. doi:10.1186/gb-2008-9-3-r60

Trotter, C. L., Gay, N. J., & Edmunds, W. J. (2005). Dynamic models of meningococcal carriage, disease, and the impact of serogroup C conjugate vaccination. *American Journal of Epidemiology, 162*(1), 89-100. doi:10.1093/aje/kwi160

Trotter, C. L., Lingani, C., Fernandez, K., Cooper, L. V., Bita, A., Tevi-Benissan, C., . . . Stuart, J. M. (2017). Impact of MenAfriVac in nine countries of the African meningitis belt, 2010-2013;15: an analysis of surveillance data. *The Lancet Infectious Diseases, 17*(8), 867-872. doi:10.1016/S1473-3099(17)30301-8

Typas, A., Becker, G., & Hengge, R. (2007). The molecular basis of selective promoter activation by the sigmaS subunit of RNA polymerase. *Mol Microbiol, 63*(5), 1296-1306. doi:10.1111/j.1365-2958.2007.05601.x

Vallenet, D., Labarre, L., Rouy, Z., Barbe, V., Bocs, S., Cruveiller, S., . . . Medigue, C. (2006). MaGe: a microbial genome annotation system supported by synteny results. *Nucleic Acids Research, 34*(1), 53-65. doi:10.1093/nar/gkj406

van Deuren, M., Brandtzaeg, P., & van der Meer, J. W. (2000). Update on meningococcal disease with emphasis on pathogenesis and clinical management. *Clin Microbiol Rev, 13*(1), 144-166, table of contents.

Vassylyev, D. G., Vassylyeva, M. N., Perederina, A., Tahirov, T. H., & Artsimovitch, I. (2007). Structural basis for transcription elongation by bacterial RNA polymerase. *Nature, 448*(7150), 157-162. doi:10.1038/nature05932

Vingadassalom, D., Kolb, A., Mayer, C., Rybkine, T., Collatz, E., & Podglajen, I. (2005). An unusual primary sigma factor in the Bacteroidetes phylum. *Mol Microbiol, 56*(4), 888-902. doi:10.1111/j.1365-2958.2005.04590.x

Virji, M. (2009). Pathogenic neisseriae: surface modulation, pathogenesis and infection control. *Nature Reviews: Microbiology, 7*(4), 274-286. doi:10.1038/nrmicro2097

Virji, M., Alexandrescu, C., Ferguson, D. J. P., Saunders, J. R., & Moxon, E. R. (1992). Variations in the expression of pili: the effect on adherence of *Neisseria meningitidis* to human epithelial and endothelial cells. *Mol Microbiol, 6*(10), 1271-1279. doi:10.1111/j.1365-2958.1992.tb00848.x

Virji, M., Heckels, J. E., Potts, W. J., Hart, C. A., & Saunders, J. R. (1989). Identification of Epitopes Recognized by Monoclonal Antibodies SM1 and SM2 Which React with All Pili of *Neisseria gonorrhoeae* but Which Differentiate between Two Structural Classes of Pili Expressed by *Neisseria meningitidis* and the Distribution of Their Encoding Sequences in the Genomes of *Neisseria* spp. *135*(12), 3239-3251. doi:doi:10.1099/00221287-135-12-3239

Virji, M., Watt, S. M., Barker, S., Makepeace, K., & Doyonnas, R. (1996). The N-domain of the human CD66a adhesion molecule is a target for Opa proteins of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Mol Microbiol, 22*(5), 929-939. doi:10.1046/j.1365-2958.1996.01548.x

Wade, J. T., Castro Roa, D., Grainger, D. C., Hurd, D., Busby, S. J., Struhl, K., & Nudler, E. (2006). Extensive functional overlap between sigma factors in *Escherichia coli*. *Nature Structural & Molecular Biology, 13*(9), 806-814. doi:10.1038/nsmb1130

Wagner, M. A., Zahrl, D., Rieser, G., & Koraimann, G. (2009). Growth Phase- and Cell Division-Dependent Activation and Inactivation of the σ(32) Regulon in *Escherichia coli*. *Journal of Bacteriology, 191*(5), 1695-1702. doi:10.1128/JB.01536-08

Wainwright, L. A., Frangipane, J. V., & Seifert, H. S. (1997). Analysis of protein binding to the Sma/Cla DNA repeat in pathogenic Neisseriae. *Nucleic Acids Res, 25*(7), 1362-1368.

Wang, L., Guo, Y., & Gralla, J. D. (1999). Regulation of Sigma 54-Dependent Transcription by Core Promoter Sequences: Role of −12 Region Nucleotides. *Journal of Bacteriology, 181*(24), 7558-7565.

Weber, H., Polen, T., Heuveling, J., Wendisch, V. F., & Hengge, R. (2005). Genome-wide analysis of the general stress response network in *Escherichia coli*: sigmaS-dependent genes, promoters, and sigma factor selectivity. *J Bacteriol, 187*(5), 1591-1603. doi:10.1128/jb.187.5.1591-1603.2005

Wigneshweraraj, S. R., Burrows, P. C., Bordes, P., Schumacher, J., Rappas, M., Finn, R. D., . . . Buck, M. (2005). The Second Paradigm for Activation of Transcription. In *Progress in Nucleic Acid Research and Molecular Biology* (Vol. 79, pp. 339-369): Academic Press.

Wong, C. n., Tintut, Y., & Gralla, J. D. (1994). The Domain Structure of Sigma 54 as Determined by Analysis of a Set of Deletion Mutants. *Journal of Molecular Biology, 236*(1), 81-90. doi:https://doi.org/10.1006/jmbi.1994.1120

Woodall, C. A. (2003). Electroporation of E. coli. In N. Casali & A. Preston (Eds.), *E. coli Plasmid Vectors: Methods and Applications* (pp. 55-59). Totowa, NJ: Humana Press.

Wörmann, M. E., Horien, C. L., Bennett, J. S., Jolley, K. A., Maiden, M. C. J., Tang, C. M., . . . Exley, R. M. (2014). Sequence, distribution and chromosomal context of class I and class II pilin genes of *Neisseria meningitidis* identified in whole genome sequences. *BMC Genomics, 15*, 253-253. doi:10.1186/1471-2164-15-253

Wörmann, M. E., Horien, C. L., Johnson, E., Liu, G., Aho, E., Tang, C. M., & Exley, R. M. (2016). *Neisseria cinerea* isolates can adhere to human epithelial cells by type IV pilus-independent mechanisms. *Microbiology, 162*(Pt 3), 487-502. doi:10.1099/mic.0.000248

Yang, Y., Darbari, V. C., Zhang, N., Lu, D., Glyde, R., Wang, Y.-P., . . . Zhang, X. (2015). Structures of the RNA polymerase-σ54 reveal new and conserved regulatory strategies. *Science, 349*(6250), 882.

Young, B. A., Gruber, T. M., & Gross, C. A. (2004). Minimal Machinery of RNA Polymerase Holoenzyme Sufficient for Promoter Melting. *Science, 303*(5662), 1382.

Yura, T. (1996). Regulation and conservation of the heat-shock transcription factor sigma32. *Genes Cells, 1*(3), 277-284.

Yura, T., Guisbert, E., Poritz, M., Lu, C. Z., Campbell, E., & Gross, C. A. (2007). Analysis of σ(32) mutants defective in chaperone-mediated feedback control reveals unexpected complexity of the heat shock response. *Proc Natl Acad Sci U S A, 104*(45), 17638-17643. doi:10.1073/pnas.0708819104

Yura, T., Nagai, H., & Mori, H. (1993). Regulation of the heat-shock response in bacteria. *Annual Review of Microbiology, 47*, 321-350. doi:10.1146/annurev.mi.47.100193.001541

Yura, T., Tobe, T., Ito, K., & Osawa, T. (1984). Heat shock regulatory gene (htpR) of *Escherichia coli* is required for growth at high temperature but is dispensable at low temperature. *Proceedings of the National Academy of Sciences of the United States of America, 81*(21), 6803-6807.

Zafar, M. A., Carabetta, V. J., Mandel, M. J., & Silhavy, T. J. (2014). Transcriptional occlusion caused by overlapping promoters. *Proceedings of the National Academy of Sciences, 111*(4), 1557.

Zaura, E., Keijser, B. J. F., Huse, S. M., & Crielaard, W. (2009). Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiology, 9*(1), 259. doi:10.1186/1471-2180-9-259

Zavascki, A. P., Fritscher, L., Superti, S., Dias, C., Kroth, L., Traesel, M. A., . . . Saitovitch, D. (2006). First Case Report of *Neisseria lactamica* Causing Cavitary Lung Disease in an Adult Organ Transplant Recipient. *Journal of Clinical Microbiology, 44*(7), 2666.

Zhang, G., Campbell, E. A., Minakhin, L., Richter, C., Severinov, K., & Darst, S. A. (1999). Crystal structure of *Thermus aquaticus* core RNA polymerase at 3.3 A resolution. *Cell, 98*(6), 811-824.

Zhang, Y., Feng, Y., Chatterjee, S., Tuske, S., Ho, M. X., Arnold, E., & Ebright, R. H. (2012). Structural basis of transcription initiation. *Science, 338*(6110), 1076-1080. doi:10.1126/science.1227786